Analytical Methods for the Identification of The Principal Cannabinoid Metabolite in Urine

NATIONAL DRUG RESEARCH CENTRE
(A UN/WHO CENTRE FOR RESEARCH AND TRAINING)
Universiti Sains Malaysia
Penang, Malaysia.

This study was funded by the United Nations Fund for Drug Abuse Control.

ISBN—967—9979-11-3

TECHNICAL RESEARCH
REPORT SERIES
1/1985

EDITOR: V. NAVARATNAM

ISBN - 967 - 9979 - 11 - 3

Published by the National Drug Research Centre
Universiti Sains Malaysia

ANALYTICAL METHODS FOR THE IDENTIFICATION AND CONFIRMATION OF THE PRINCIPAL CANNABINOID METABOLITE IN URINE*

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*THIS STUDY HAS BEEN FUNDED BY THE UNITED NATIONS FUND FOR DRUG ABUSE CONTROL (UNFDAC)

ACKNOWLEDGEMENT

1. Tan Sri Datuk Rozhan bin Kuntom, Secretary-General, Ministry of Home Affairs, for his support and co-operation in the execution of this project.

2. Datuk (Dr.) Khalid bin Sahan, Director-General, Ministry of Health, for his interest.

3. Datuk Musa bin Mohamad, Vice-Chancellor and Chairman National Drug Research Centre, for his advice and support.

4. Professor (Dr.) G. Di-Gennaro, Executive Director United Nations Fund for Drug Abuse Control, for his interest and support.

5. The Deputy Vice-Chancellors, Universiti Sains Malaysia, for their kind interest.

6. The National Institute for Drug Abuse for the assistance in providing the reference materials.

7. The Anti-Dadah Task Force, for their assistance.

8. Dr. Chandra Jayaram and Mr. Tan Soo Choon, for their advice and criticisms.

9. Mr. S. Vijayan, Final Year Student in Chemistry, for participating in the study as a research assistant during the University vacations.

10. The laboratory technical and secretarial staff of the National Drug Research Centre for all their assistance during the study and in the preparation for this report.

Kaedah-Kaedah Analitis untuk Pengenalan Kannabinoid Asas pada Air Kencing

Dadah psikoaktif utama pada kanabis ialah tetrahidrokanabinol (THC) hadir sebagai asid karboksiliknya, asid tetrahidrokanabinolik (THCA). Ia merupakan hampir 5% dari berat tumbuhan kanabis, dan boleh menjadi lebih tinggi bagi pelbagai penyediaan kanabis misalan hashish.

Bila dihisap dengan rokok, THCA mengalami pendekarboksilan kepada THC yang mana kemudian diserap ke dalam aliran darah dan seterusnya mengakibatkan kesan psikotomimetik. THC mengalami metabolisme untuk menghasilkan terbitan-terbitan hidroksil dan karboksilnya. Metabolit-metabolit dikumuhkan melalui air kencing dan najis.

Metabolit utama THC ialah 11-nor-THC-9-COOH (THC-COOH). Ia wujud pada aliran darah sebaik sahaja dihisap dengan kannabis rokok dan kesan maksimanya dirasai selepas 40 minit. Takatnya kemudian turun perlahan-lahan untuk 7 hingga 10 hari berikutnya. THC-COOH wujud pada air kencing dalam masa 2 jam dari waktu ia dihisap dan dikumuhkan berterusan selama 1 minggu. THC-COOH dikumuhkan sebagai glukoronidanya.

Teknik-teknik analitis untuk penentuan pengambilan kanabis yang terbaru adalah dikembangkan untuk penentuan THC-COOH, daripada TLC, dalam cecair biologi.

Dalam kajian ini kaedah-kaedah kromatografi lapisan-nipis (TLC), kromatografi gas (GC) dan kromatografi gas - spektrofotometer jisim (GCMS), telah diperkembangkan untuk analisis THC-COOH dalam air kencing. Sebelum sebarang teknik kromatografi di atas dapat digunakan

cara-cara yang sesuai untuk pengekstrakan dan penerbitan THC-COOH perlu dikembangkan. Setiap kaedah ini, had pengenalannya dan hasilnya dipersembahkan di sini.

3 ml air kencing digunakan untuk kaedah analitis. Air kencing dihidrolisiskan dahulu di bawah keadaan basik untuk membelah ikatan glukoronida pada THC-COOH. Air kencing yang telah dihirolisiskan (pada pH yang tinggi), separa diceriahkan melalui pengekstrakan eter. Air kecing itu kemudian diasidifikasikan dan THC-COOH padanya diekstrakkan dengan sikloheksana-etilasetat (96:4). Ekstrak organik itu diceriakan lagi melalui suatu kaedah pengekstrakan-balik. Ekstrak organik itu kemudian dikeringkan dibawah nitrogen.

Residu kering yang dihasilkan, dilarutkan dalam suatu isupadu kecil aseton dan dilakukan TLC di atas plat silika gel $60GF_{254}$, yang sedia terselaput, dan dikembangkan dengan kloroform:metanol:amonia (70:30:2). Bila plat TLC itu disemburkan dengan larutan 'Fast Blue B', THC-COOH kelihatan sebagai suatu bintik merah muda pada Rf 0.31. Sampel-sampel air kencing yang mengandungi kepekatan TLC melebihi 75 ng ml^{-1} dapat dikesan dengan GC.

Penerbitan (sililasi) ekstrak air kencing adalah perlu untuk analisis GC atau GCMS. Sililasi dilakukan dengan menggunakan BSTFA dalam asetonitril (2:1). Vial mikro yang mengandungi ekstrak yang telah dilarutkan dengan BSTFA-asetonitril ditutup dan dipanaskan pada suhu 90°C selama 90 minit. Ekstrak yang telah diterbitkan adalah sedia untuk analisis.

Analisis GC dilakukan dengan menggunakan suatu turus OV-101 dengan alat pengesan FID. Gas pembawa adalah nitrogen. Keadaan-keadaan untuk analisis adalah dicatit dibawah:

Suhu permulaan tanur:	250°C
Masa permulaan tanur:	10 minit
Suhu akhir tanur:	280°C
Masa akhir tanur:	5 min
Kadar suhu tanur:	5°C per minit
Suhu Injektor	300°C
Suhu Detektor (pengesan)	300°C
Aliran gas pembawa	20 ml min^{-1}

Terbitan trimetilsilil THC-COOH (THC-COOTMS) memberi suatu puncak GC pada 5.75 minit. Sampel-sampel air kencing yang mengandungi 20 ng ml^{-1} THC-COOH telah dapat dikesan dengan GC.

Analisis GCMS telah dilakukan dengan mod "mass scanning" dan mod 'single ion monitoring' yang lebih peka. Turus OV-101 telah digunakan. Keadaan-keadaan untuk GCMS adalah dicatit di bawah:

Suhu permulaan tanur:	250°C
Masa permulaan tanur:	5 minit
Suhu akhir tanur:	280°C
Masa akhir tanur:	2 minit
Kadar suhu tanur:	10°C min^{-1}
Suhu injektor	280°C
Aliran gas pembawa helium:	20 ml min^{-1}

Masa retensi untuk THC-COOTMS dengan GLMS ialah 1.5 minit. Identitinya telah dikenalpastikan dari spektrum jisimnya, yang telah memberi ion molekular pada m/e 489 dan puncak asas pada m/e 371 bersamaan dengan kehilangan kumpulan COOTMS. Sampel-sampel air kencing yang mengandung 20 ng ml^{-1} THC-COOH telah dapat dikesan dengan kaedah GCMS di atas.

Kehilangan yang dialami semasa kaedah pengekstrakan telah dianggarkan melalui pengukuran kepekatan kanabinoid dalam sampel-sampel air kecing sebelum pengekstrakan dan sekali lagi pada ekstrak yang dibentuksemula. (Pengukuran ~~telah dibuat~~ menggunakan kaedah EMIT. Keputusan yang diperolehi telah menunjukkan kehilangan 5 ng ml^{-1}, yang mana tidak bergantung kepada kepekatan asal. Kehilangan ini terjadi semasa langkah-langkah pemindahan fasa pada kaedah pengekstrakan tersebut.

Kehilangan yang dialami semasa penerbitan boleh dianggarkan daripada plot luas puncak GC relatif lawan kepekatan asal pada air kencing.

Dari ini suatu kehilangan sebanyak 12.5 ng ml^{-1} telah diperolehi untuk keseluruhan proses dan oleh itu suatu kehilangan 5.5 ng ml^{-1} untuk langkah penerbitan. Kehilangan ini mungkin disebabkan oleh pengendalian sampel (cf kehilangan semasa pengekstrakan) dan oleh terikan sterik terhadap sililasi lengkap THC-COOH.

Keadah EMIT untuk analisis kanabinoid telah diujikan terhadap kaedah kromatografik di atas. Peralatan EMIT itu dibekalkan dengan tiga kalibrator bersamaan dengan 3 kepekatan THC-COOH berlainan dalam air kencing:-

Kalibrator Negatif	0 ng ml^{-1}
Kalibrator Rendah	20 ng ml^{-1}
Kalibrator Pertengahan	75 ng ml^{-1}

Sampel yang memberikan respon EMIT yang sama atau lebih dari respon Kalibrator Rendah boleh dianggap positif. Sebaliknya sampel dengan respon rendah dari Kalibrator Rendah dianggap negatif. Sampel-

sampel air kencing yang telah diuji dengan EMIT boleh dibahagikan kepada 3 kategori. Satu sampel sahaja telah memberi respon negatif EMIT tetapi telah didapati positif dengan GC dan GCMS. Respon EMIT untuk sampel ini telah didapati hampir sama dengan respon Kalibrator Rendah. Lima sampel telah memberi nilai EMIT di antara bacaan Kalibrator Rendah dan Pertengahan. Sampel-sampel ini telah dibuktikan positif dengan GC dan GCMS. Enam sampel yang telah memberi bacaan EMIT lebih dari respon Kalibrator Pertengahan telah dibuktikan oleh TLC, GC dan GCMS.

Adalah dicadangkan supaya EMIT digunakan sebagai suatu kaedah pentaksiran sampel air kencing yang disyaki mengandungi kananbinoid. Cadangan ini adalah berdasarkan "turnover" sampel yang tinggi kaedah EMIT bersama dengan keputusan perbandingan pada kajian ini.

Sampel-sampel yang telah memberikan respon EMIT yang rendah daripada takat Kalibrator Rendah boleh dianggap negatif dan tidak memerlukan pengujian selanjutnya. Sampel-sampel yang memberikan bacaan antara Kalibrator Rendah dan Pertengahan boleh dibuktikan dengan GC atau GCMS. Sampel-sampel dengan respon yang lebih dari Kalibrator Pertengahan hanya perlu pembuktian TLC.

Dalam kajian ini, kaedah-kaedah telah dikembangkan untuk pengekstrakan, penerbitan dan kromatografi (TLC, GC dan GCMS) THC-COOH dalam 3 ml air kencing. Kaedah-kaedah pengekstrakan dan penerbitan adalah mudah secara perbandingan dan masa analisis kromatografi pendek. Kaedah EMIT telah di bandingkan dengan kaedah-kaedah di atas dan telah didapati sesuai untuk tujuan pentaksiran.

CHAPTER 1

GENERAL BACKGROUND

1.1 Introduction

Cannabis is obtained from the flowering tops and dried leaves of the hemp plant CANNABIS SATIVA L, in particular from the resin produced by the flowering tops. Many preparations of the drug are in common use, varying in potency.

The terminology used for the various preparations are different and vary from region to region. A list of some of the more commonly used terms and a description is provided below[1].

MARIJUANA or POT or GRASS:	Dried flowering tops of the plant.
HASHISH or CHARAS:	Unadulterated resin from flowering tops of cultivated female plants.
HASH OIL:	Extracts obtained from the resin (flowering tops).
BHANG:	Tops of uncultivated female plants, boiled in water or milk. Drunk, or dried and smoked.
GANJA:	Similar to bhang, but obtained from cultivated plants.

The order of potency of the above preparations is:-

Marijuana = Bhang < Ganja < Hashish < Hash oil

The consumption of cannabis, now thought to be the most widely used of all illicit drugs, is mainly by smoking, occassionally orally and rarely intravenously.

1.2 Chemistry

The compounds associated with the pharmacological effects of the drug are the cannabinoids, of which over 50 have so far been identified. The main cannabinoids are (Figure 1):-

TETRAHYDROCANNABINOL (Δ^9-THC or just THC in this report)
CANNABIDIOL (CBD)
CANNABINOL (CBN)

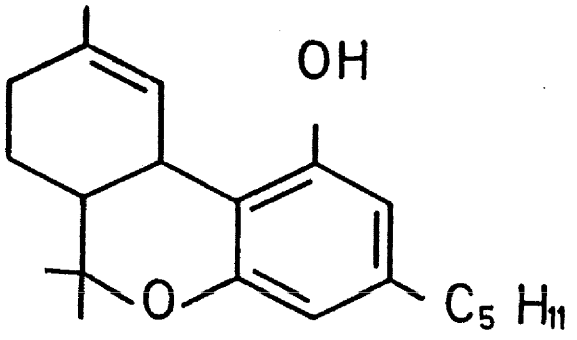
and to a lesser extent (Figure 2):-

CANNABIGEROL (CBG)
CANNABICYCLOL (CBC)
CANNABICHROMENE (CBCh)

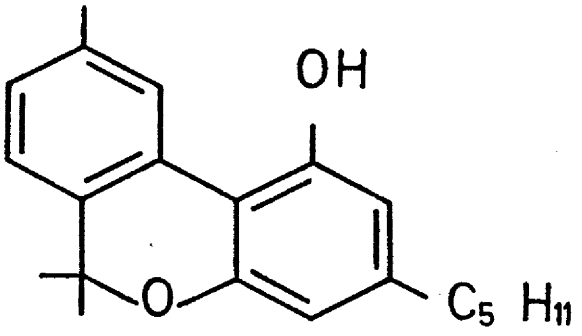
THC, CBN, CBD and some other cannabinoids are present in fresh plant material in the form of their carboxylic acids (Figure 3):-

Δ^9-TETRAHYDROCANNABINOLIC ACID (Δ^9-THCA, THCA)
CANNABINOLIC ACID (CBNA)
CANNABIDIOLIC ACID (CBDA)

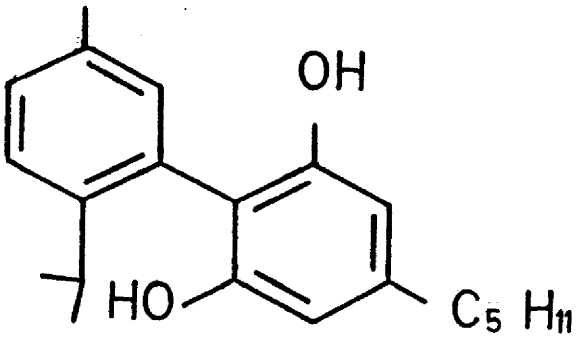
However these acids are very unstable and during storage (light and heat) or when the drug is smoked (heat), they rapidly decarboxylate to release the parent cannabinoids. Although the stability of the decarboxylated cannabinoids is high, THC slowly decomposes further in the presence of light and air (oxygen) to CBN[2]. The effect of light has been shown to be greater than oxygen on its decomposition[3]. At 80°C, 50% of THC is converted to CBN in 4 days. The relative proportions of the cannabinoids varies depending on the source (geographical and genetic) of the plant. In some varieties (e.g. from Thailand) CBD may be entirely absent[4]. Some samples are rich in THC and practically devoid of other cannabinoids and others (mainly those grown in cooler climates such as in



THC

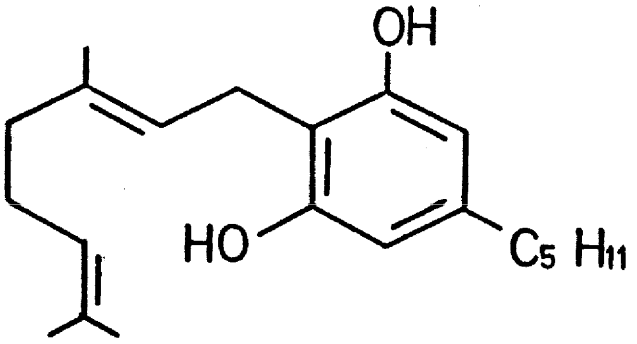


CBN

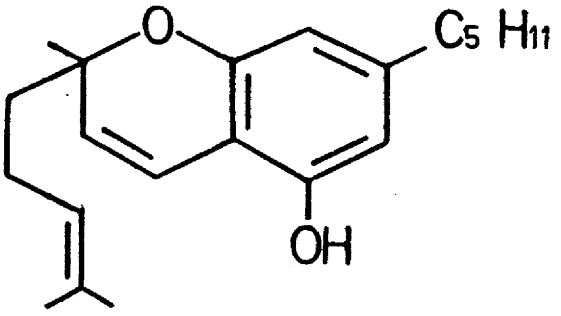


CBD

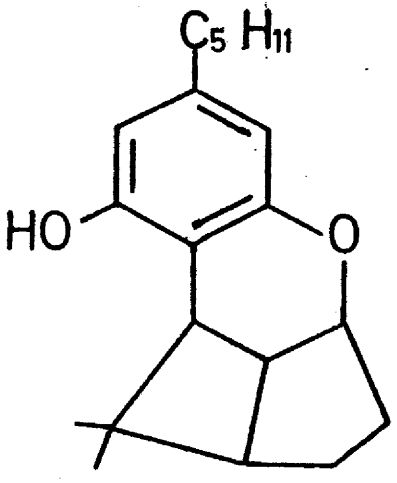
FIGURE 1 **The Principal Cannabinoids of Cannabis**



CBG

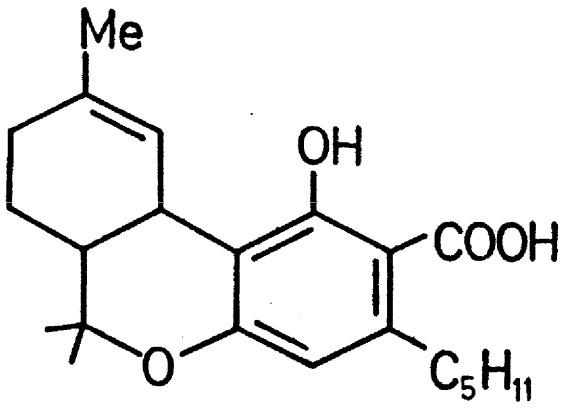


CBCh

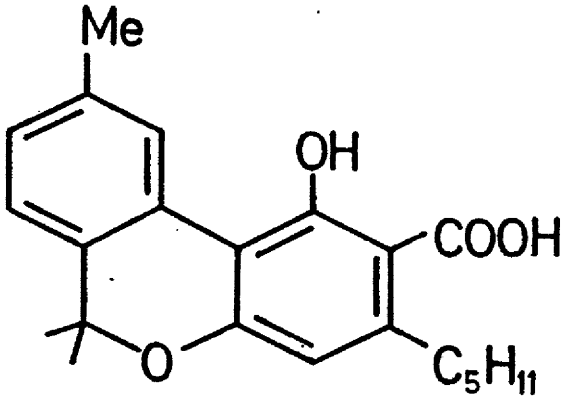


CBC

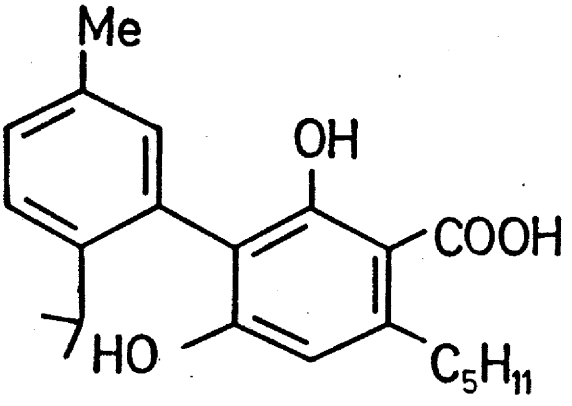
FIGURE 2 The Minor Cannabinoids of Cannabis



THCA



CBNA



CBDA

FIGURE 3 **The Principal Cannabinoids as their Carboxylates**

Europe) tend to have lower concentrations of THC but much higher levels of the relatively inactive CBD.

THC is the principal cannabinoid and is responsible for most of the characteristic psychological effects of cannabis. It is present to an extent varying from 2-40%, depending on the nature of drug preparation[5]:-

Marijuana: 5% THC max.

Hashish: 10% THC max.

Hash oil: 35% THC max.

The potency of the drug preparation thus depends on the amount of THC present. Consequently the price of cannabis on the illicit market is governed by its quality as reflected by the level of THC present[6].

The freshness of a sample of cannabis is determined by the ratio of its THC to CBN content. The higher this ratio, the fresher the sample, since THC degrades to CBN[7].

Apart from THC, other cannabinoids may contribute to its activity through interactions or direct effects on certain specific pharmacologic measures[8]. The activity of either CBD or CBN is less than 10% of THC.

1.3 Pharmacology

Cannabis has potentially therapeutic effects[9] such as

- Reduction of intraocular pressure
- Bronchodilation
- Anticonvulsant action
- Retardation of tumour growth

However the psychotomimetic effects of cannabis have led to its widespread abuse with societal and public health implications as indicated below:

i. - Traffic accidents: Distortion of perception and time.

ii. - Carcinogenic potential: Smoke condensate contains considerably higher amounts of polynuclear aromatic hydrocarbons than tobacco smoke.

iii. - Cardiovascular effects: Hypotension. Tachycardia. Reduction of blood flow to the brain.

1.4 Metabolism

Smoking is the most effective way of achieving the effects of THC since its low solubility (in aqueous media) results in slow absorption through the intestinal tract if taken orally. Inhalation results in a rapid rise in the blood THC level. Pharmacological effects occur within 2-3 minutes of smoking, with the maximum effect within 20-30 minutes, and lasting 90-120 minutes (Figure 4). When ingested, the time scale is expanded.

The physiological "high" induced by the drug tends to lag behind the maximum blood levels. This lag can be attributed to two causes:-

i. Rate of penetration of the drug into the tissues such as brain;

ii. Contribution by active metabolites.

During smoking, only 50% of the THC content of the cigarette gets absorbed into the body. The remaining 50% is exhaled. Of the absorbed THC, 99% gets metabolised and only 1% is excreted unchanged. Seventy-two hours after smoking, 50% of the inhaled THC would have been excreted (as metabolites). The remaining 50% is distributed throughout the body where it is absorbed by fatty tissue and excreted slowly over the next 10 days. Excretion is mainly via urine (25%) and faeces (65%).

Cannabinoids are readily metabolised, and about 80 metabolites have so far been identified[10]:-

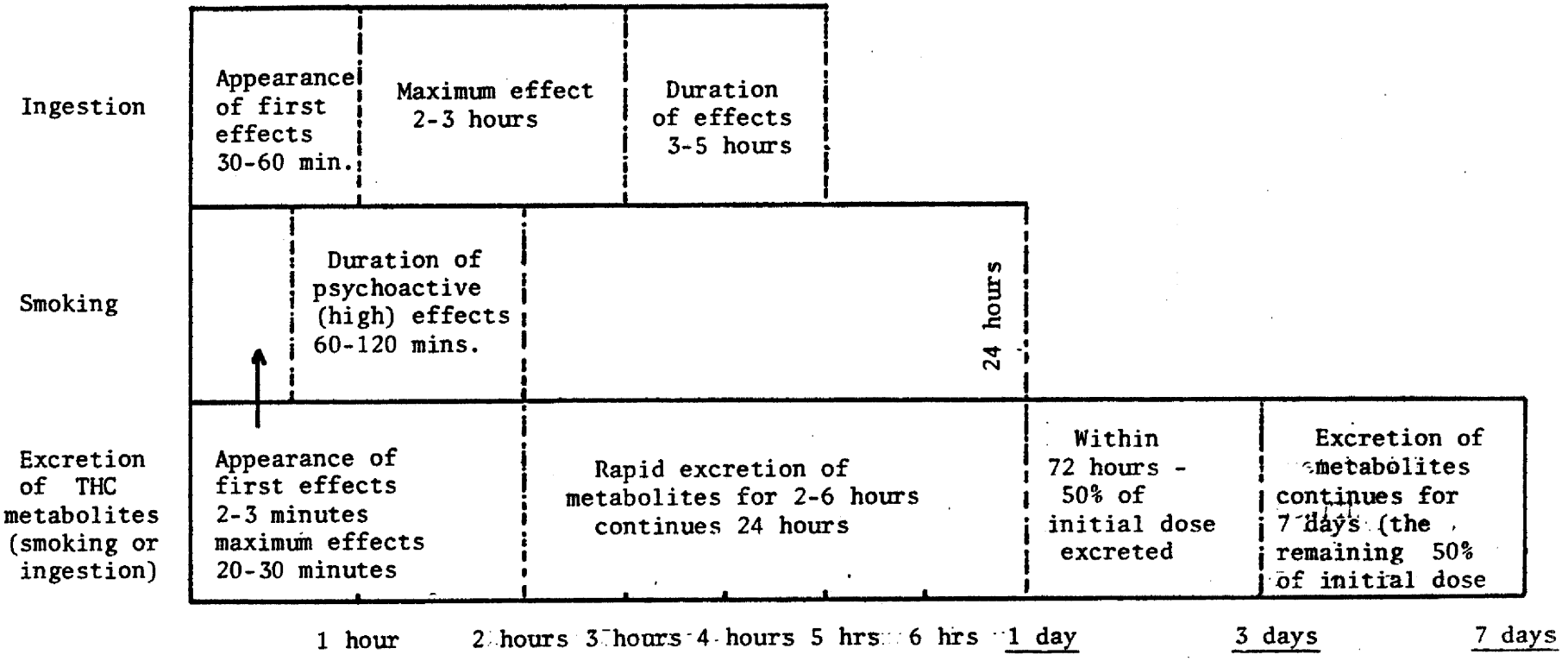


Figure 4

A comparison, over time, of the reported effects (smoking or ingestion) versus excretion of - THC metabolites

THC - 35 metabolites

CBD - 22 metabolites

CBN - 22 metabolites

The types of reaction which they are subjected to include (Figure 5):

i. oxidation to acids, aldehydes and ketones

ii. conjugation with glucuronic acid or fatty acids

iii. epoxidation of double bond

Glucuronide formation may occur at either the carboxyl or hydroxyl positions, or directly to the aromatic ring.

Initial metabolism of cannabinoids in marijuana smoke takes place in the lungs. Metabolism of orally ingested cannabis occurs in the liver.

The major lung metabolites are formed via side chain hydroxylation. The major liver metabolites are hydroxylated derivatives of the cyclohexene ring (Figure 6).

The main metabolite excreted is the glucuronide conjugate of 11-nor-THC-9-carboxylic acid (THC-COOH). The precursor of THC-COOH is 11-hydoxy-THC (THC-OH). THC-OH has psychotomimetic activity comparable to that of THC[11] and is considered by some to be the active component instead of THC. THC-OH is short-lived and rarely present in urine. About 80% of the excreted THC-COOH is in the form of its glucuronide conjugate[11].

The concentration of THC-COOH in plasma rapidly reaches a maximum 20-40 minutes after smoking (Figure 7) and soon exceeds that of

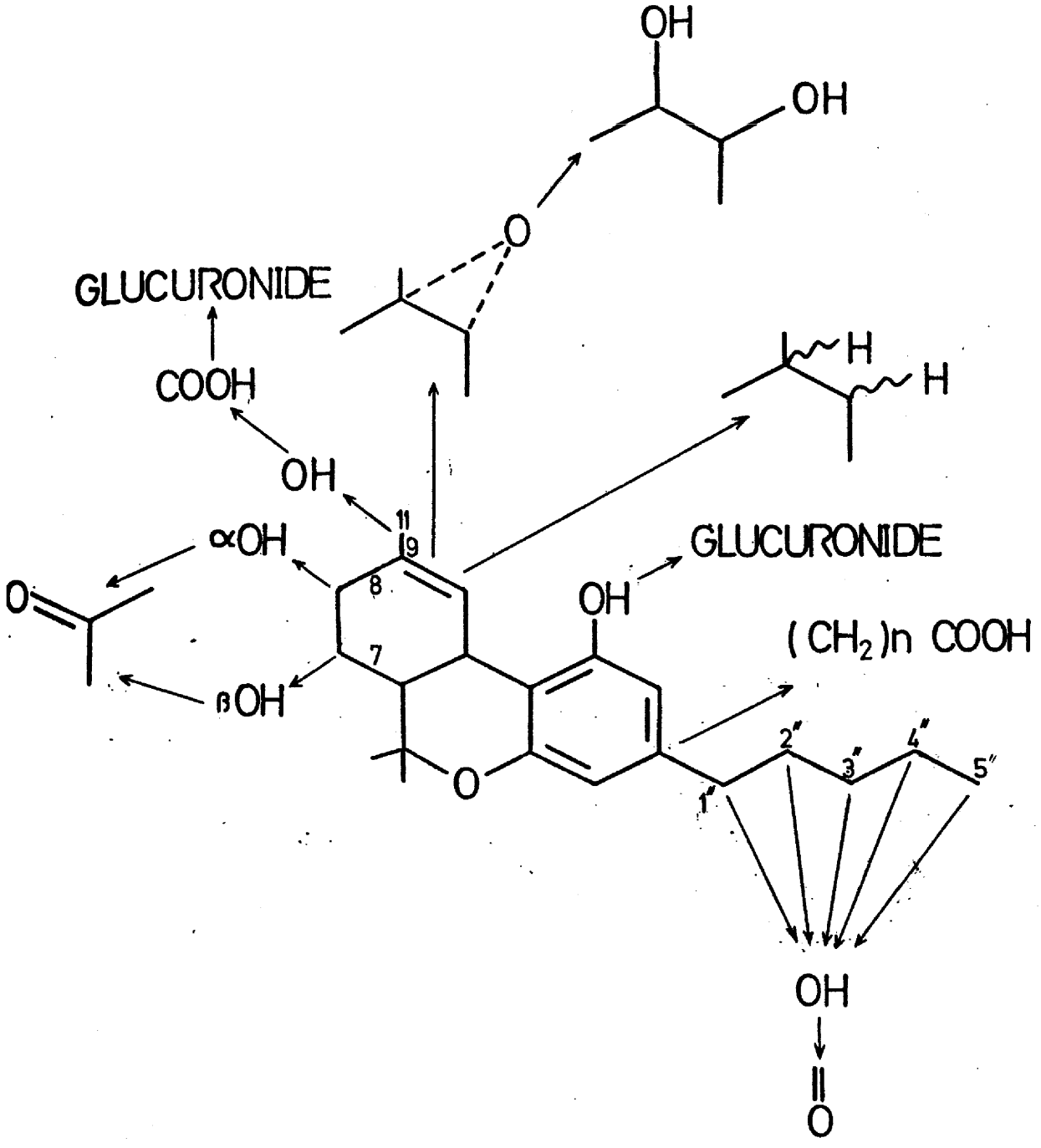
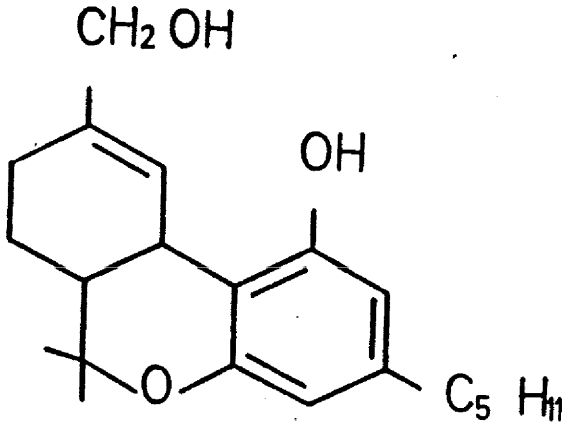
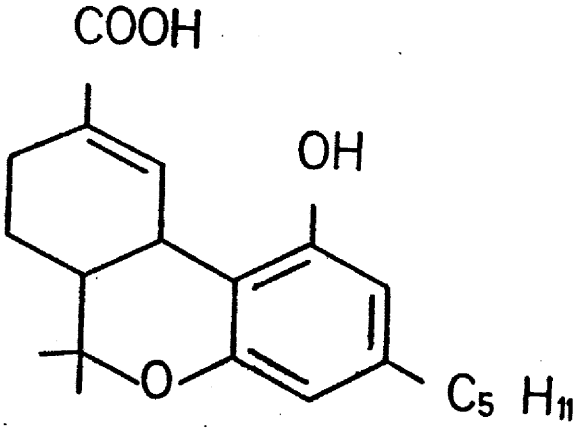


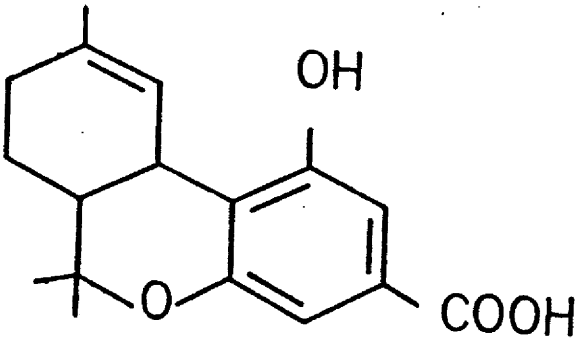
FIGURE 5 **The THC Metabolic Routes**



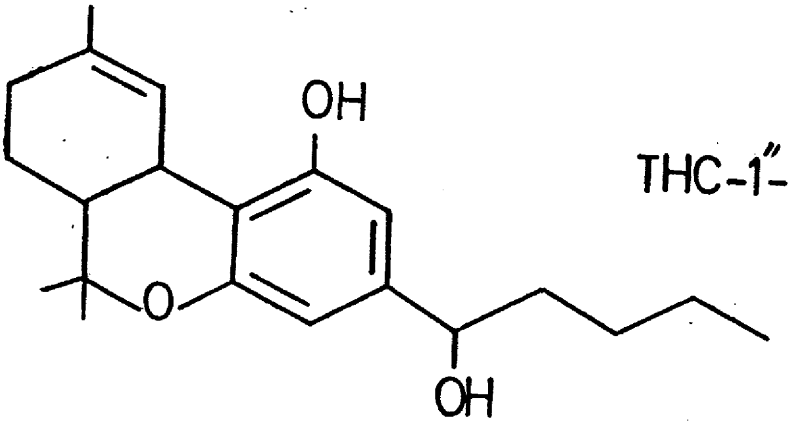
THC-11-OH



THC-9-COOH

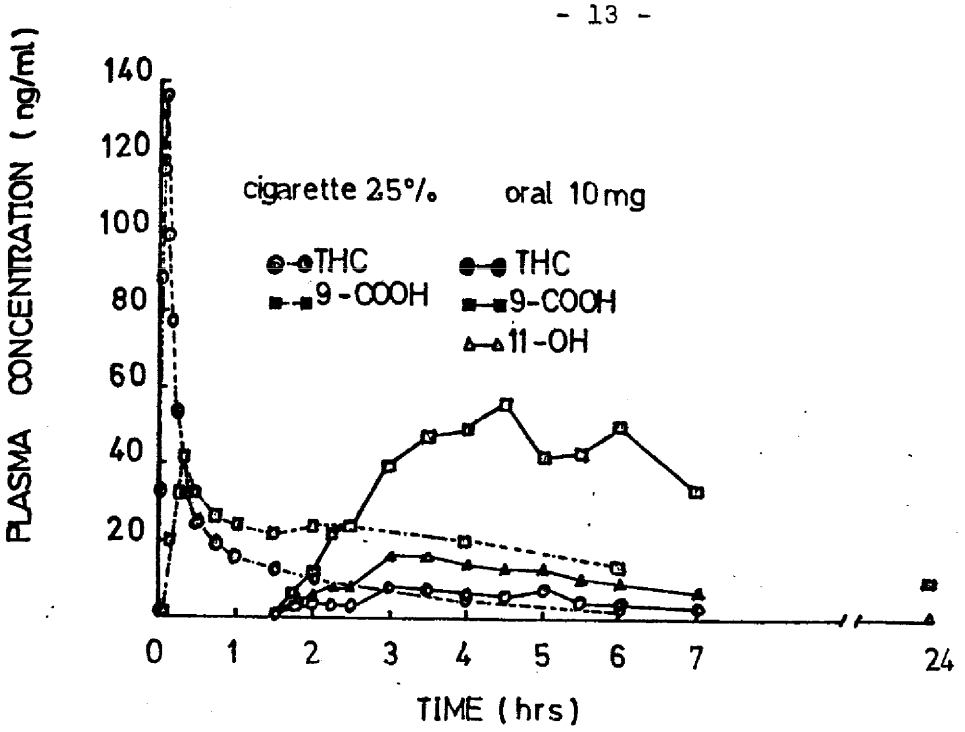


THC-3′-COOH



THC-1″-OH

FIGURE 6
The Main Metabolites of THC



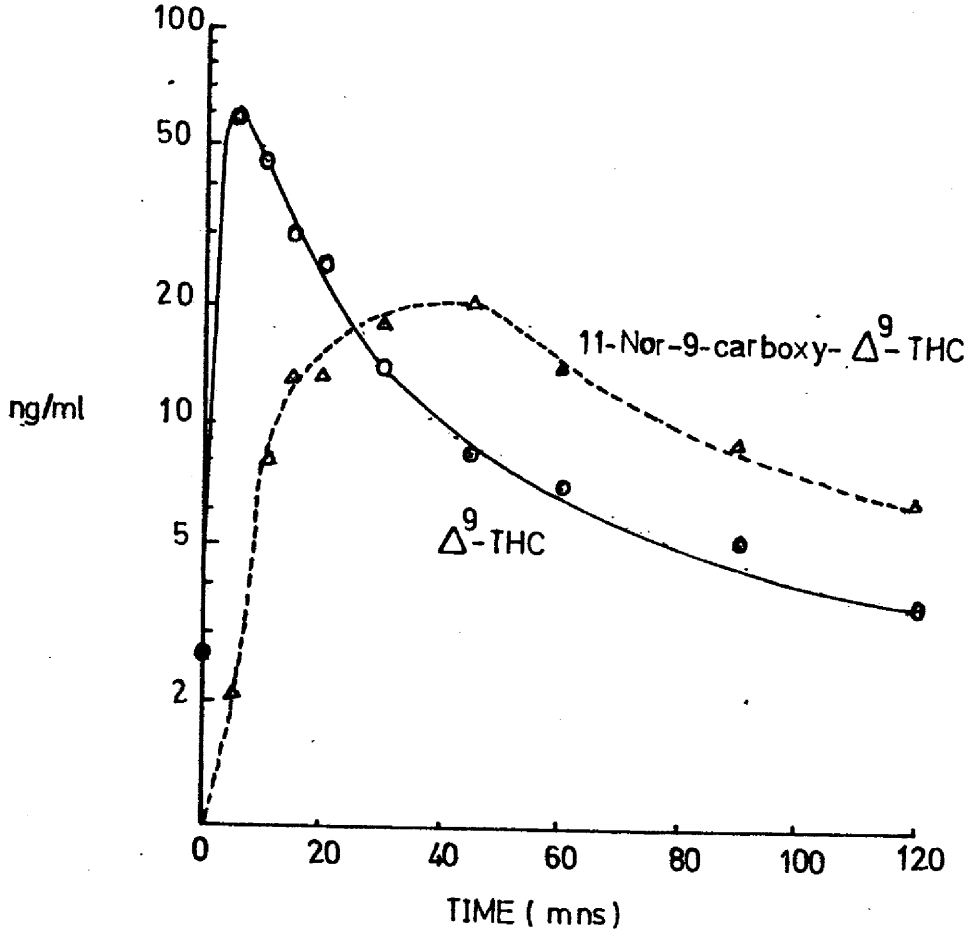


FIGURE 7 **The Pharmacokinetics of THC and its Metabolites**

THC. This rapid rise is followed by a slow decline and can be measured in the body for a longer period of time. THC is rapidly eliminated up to 40 minutes followed by slow elimination over 24 hours. During this period, the concentration of THC-OH is 1/25th that of THC[12]. For orally ingested THC, the blood THC and THC-COOH levels take longer to peak, and the levels are lower. This is because the ingested THC has to first pass through the liver which is a major detoxification organ in the body.

Since THC-COOH is the principal cannabinoid that is elminated by the body and over a reasonable length of time, its detection in body fluids would act as the best indicator of recent cannabis consumption.

CHAPTER 2

REVIEW OF ANALYTICAL METHODS

Earlier methods of detecting cannabinoids in body fluids relied on the identification of THC and not THC-COOH. Only more recently, after the realisation that the major excreted cannabinoid is THC-COOH, were methods developed for its detection. These methods were geared to detect cannabinoids in either blood, urine or both.

The most popular method used for the detection of THC in plasma in the early 1970's was radioimmunoassay. More recently the trend has been towards the detection of THC-COOH in urine using gas chromatography linked to mass spectrometry (GCMS). The main reasons for the latter methodology were increased detectability (THC-COOH vs THC) and better specificity (THC-COOH vs total cannabinoid activity).

The two major body fluids used for the detection of cannabinoids are blood (plasma) and urine. To a lesser extent saliva, faeces, bile and body tissue (liver) have also been used. Urine is preferred over plasma from the point of view of collection and handling.

The analytical methods used can be divided into two major groups:-

Immunological	- 1. Radioimmunoassay (RIA)
	- 2. Enzyme Multiplied Immunoassay Technique (EMIT)
Chromatographic	- 3. Thin Layer Chromatography (TLC)
	4. High Performance Liquid Chromatography (HPLC)
	5. Gas Chromatography (GC)
	6. Gas Chromatography - Mass Spectrometry (GCMS)

The various methods differ in sample preparation, analytical procedure, duration, sensitivity and selectivity. A summary of most of the methods that have been published over the decade are presented in Table 1.

The following account of the above immunological and chromatographic methods in existence is an elaboration of the information provided in Table 1.

TABLE 1

A Summary of Existing Analytical Procedures for the Detection of Cannabinoids in Biological Fluids

REF.**	CANNABINOID	MATRIX	EXTRACTION PROCEDURE	ANALYSIS	DETECTION	SENSITIVITY	DERIVAT. AGENT	COMMENTS
9	THC	PL	Hex	GC CIMS	SIM	0.2 ng ml^{-1}	BSTFA-TMCS	NH_3=Rgt. gas
	THC-OH & THC-COOH	PL & UR	Hex-EtOAc	GC CIMS	SIM	-	BSTFA-TMCS	-
9*	THC	PL	Pet. ether MeOAc-EtOH	TLC	FL, ^3H, ^{14}C	few ng ml^{-1}	14C Dansyl Chloride	-
	THC & Metabolites	PL	NA	TLC	FL	0.2 ng ml^{-1}	2-p-chloro sulphonyl -3-phenyl-indone	Unstable derivative. Measure within few minutes of TLC
	THC	PL	NA	HPLC-RIA	-	0.1 ng ml^{-1}		-
	Cannabinoids	PL	NA	GC	NPD	0.5 ng ml^{-1}	Diethyl phosphate	-
	-	PL	NA	PC	-	0.5 ng ml^{-1}	-	-

(cont.)

REF.**	CANNABINOID	MATRIX	EXTRACTION PROCEDURE	ANALYSIS	DETECTION	SENSITIVITY	DERIVAT. AGENT	COMMENTS
10 (10=23)	THC-COOH	UR	i. OH^- ii. cycHex-EtoAc	TLC	VIS	50 ng ml^{-1}	FBB-RR (pink)	100% correlation with EMIT
12	THC, CBN & THC-OH	PL	i. Pet. ether ii. Sephadex LH-20	GC EIMS	-	0.1 ng ml^{-1}	BSTFA-TMCS	Useable lower limit for EI & CI is 1 ng ml^{-1}
			Hex	GC CIMS	-	0.5 ng ml^{-1}	PFPA	Isobutane is carrier gas
	-	-	Hex	GC	ECD	-	PFPA	Double column Packed+Capillary
13	THC	UR	NA	RIA	^{14}C, ^{3}H	0.25 ng	-	No interference in urine
14	THC	PL	-	RIA	^{3}H	5 ng ml^{-1}	-	Possible interference in plasma. Performance subject to buffer conditions also

(cont.)

REF.**	CANNABINOID	MATRIX	EXTRACTION PROCEDURE	ANALYSIS	DETECTION	SENSITIVITY	DERIVAT. AGENT	COMMENTS
15	THC and metabolites	PL & UR	-	RIA	^{125}I	2 ng ml^{-1}	-	
16	THC	PL	-	RIA	^{125}I	2.5 ng ml^{-1}	-	^{125}I is a better radioligand than ^{14}C or ^{3}H
		-	-	-	^{3}H	5 ng ml^{-1}	-	-
	THC-COOH	PL	-	RIA	^{125}I	20-50 ng ml^{-1}	-	
17	THC	PL	MeOH	i. HPLC ii.RIA	UV -	5 ng ml^{-1} 1 ng ml^{-1}	- -	- -
	THC-COOH	UR	i. OH^- ii. MeOH	i. HPLC ii.GCMS	UV -	- -	- -	- -
18	THC & metabolites	UR	MeOH (OH^-)	HPLC-RIA	UV	-	-	-
21	THC-COOH	UR	i. Enzyme ii.Ether	TLC	VIS	80 ng	FBB(Magenta)	FBB cross reacts with rosemary, thyme and sage

(cont.)

REF.**	CANNABINOID	MATRIX	EXTRACTION PROCEDURE	ANALYSIS	DETECTION	SENSITIVITY	DERIVAT. AGENT	COMMENTS
22	THC-COOH	UR	i. OH^- ii. BPA iii. Acetone	TLC	VIS	20 ng ml^{-1}	FBB-RR (Red)	
24	THC-COOH	UR	i. OH^- ii. Hex-EtOAc	GC	FID	20 ng ml^{-1}	TMAH-DMSO -MeI	Results checked by GCMS
25	THC-COOH & othe canna-binoids	PL	MeOH	EMIT	40 ng ml^{-1} (10 ng ml^{-1})	-	-	Detectable down to 10 ng ml^{-1}
26	THC-COOH	PL & UR	Ether	GCMS	-	few ng ml^{-1}	i. CH_2N_2 ii. BSA	Purification by Sephadex LH-20 after methylation
27	THC-COOH	PL & UR	Hex-EtOAc	GCCIMS	-	0.1 ng ml^{-1}	i. BF_3-MeOH ii. TFAA	Capillary GC in combination with negative ion CIMS. TFA derivative - eletron capturing. BF_3-MeOH cleaner than CH_2N_2.
	THC-OH	-	-	-	-	0.5 ng ml^{-1}	TFAA	
	THC	-	-	-	-	0.2 ng ml^{-1}	TFAA	

(cont.)

REF.**	CANNABINOID	MATRIX	EXTRACTION PROCEDURE	ANALYSIS	DETECTION	SENSITIVITY	DERIVAT. AGENT	COMMENTS
28	THC-COOH	UR	i. OH^- ii. Bond-elut iii. MeCN	HPLC	UV	25 ng ml^{-1}	-	No derivatisation necessary for HPLC (cf. GC)
29	THC	FE	Pet. ether	GCMS	-	-	BSTFA	-
	THC-COOH	UR	i. Enzyme ii. Ether	GCMS	-	-	BSTFA	-
30	THC-COOH	UR	i. Enzyme ii. Et_2O-Hex	HPLC GC GCCIMS	UV ECD	20 ng ml^{-1}	PFPA-PFPOH	80% recovery Methane=Rgt.gas
31	THC	PL	Pet. ether -iPeOH	GCMS	-	-	BSTFA	-
32	THC	PL	Hex	GCCIMS	SIM	0.2 ng ml^{-1}	BSTFA-TMCS	NH_3=Rgt. gas
	THC & THC-OH	PL	i. Pet. ether +iAmyOh ii. Sephadex LH-20	GCCIMS	SIM		BSTFA	
	THC, THC-OH & THC-COOH	PL	Hex-EtoAc	GCCIMS	SIM		i. CH_2N_2 ii. BSA	

(cont.)

REF.**	CANNABINOID	MATRIX	EXTRACTION PROCEDURE	ANALYSIS	DETECTION	SENSITIVITY	DERIVAT. AGENT	COMMENTS
33	THC-OH & THC-diOH	TIS	Hex-Pet.ether	TLC	-	-	-	-
	THC & THC-OH	UR	NA	GCMS	-	0.2 ng	BSA	-
34	THC	UR & PL	Hex	HPLC TLC	UV FL	1 ng 15 ng	Dabsylchloride Dabsylchloride	- -
35	THC	SL & PL	Pet.ether-MeOAc	TLC+MS	FL	Sub-nanogram	Dansyl chloride	THC is detectable in saliva upto 2 hours after smoking
36	THC	PL	Hex-iAmyOH	TLC	FL	0.1 ng (0.4 ng ml^{-1})	2-p-chloro-sulphonyl-3-phenylindone	Spray with sodium methoxide. Green Fluorescence
37	THC-COOH	UR	i. OH^- ii. cycHex-EtOAc iii. Na_2SO_4	TLC	VIS	-	FBB	Urine extract purified through Na_2SO_4 column
	-	-	-	GCMS	-	-	TMAH-DMSO-MeI	-

cont.)

REF.**	CANNABINOID	MATRIX	EXTRACTION PROCEDURE	ANALYSIS	DETECTION	SENSITIVITY	DERIVAT. AGENT	COMMENTS
39	THC & THC-OH	PL	Hex	HPLC	EChD	5 ng ml^{-1}	-	-
40	THC	PL	-	HPLC + GC	ECD	1 ng ml^{-1}	PFBB	HPLC removes interferng contaminants
	-	-	-	HPLC + RC	^{14}C	0.2 ng ml^{-1}	-	-
41	THC	PL	Pet.ether	HPLC MS	UV SIM	1 ng ml^{-1}	-	-
	-	BR	Polyethylene wafer- MeOH	-	-	-	-	-
	-	SL, BL & BN	Pet. ether	-	-	-	-	-
42	THC	PL	Hex	GC	ECD	1 pg (std) 500 pg ml^{-1}	PFPA	Double column GC removes interfering substances
43	THC-COOH	UR	i. OH^- ii. Hex-EtOAc	GC	ECD	1 ng ml^{-1}	PFBB	Sensitive and fast method

(cont.)

REF.**	CANNABINOID	MATRIX	EXTRACTION PROCEDURE	ANALYSIS	DETECTION	SENSITIVITY	DERIVAT. AGENT	COMMENTS
44	THC	PL	Hex	GC CIMS	SIM	0.5 ng ml^{-1}	BSTFA-TMCS	Methane=Rgt. gas
45	THC & metabolites	PL	Hex	GCMS	MIM	5 pg ml^{-1}	BSTFA	-
46	THC	PL	-	RIA	^{125}I	1.5 ng ml^{-1}	-	-
47	THC & THC-OH	PL	-	RIA	^{3}H	2 ng ml^{-1}	-	-
48	THC & THC-COOH	UR & PL	-	RIA	^{3}H	-	-	-

KEY (TABLE 1)

BF_3	Boron trifluoride
Bl	Bile
BN	Brain
BPA	Bonded phase adsorption
BR	Breath
BSA	Bis trimethylsilyl acetamide
BSTFA	Bis trimethylsilyl trifluoro acetamide
CBN	Cannabinol
CI	Chemical ionisation
cycHex	Cyclohexane
DMSO	Dimethylsulphoxide
ECD	Electron capture detector
EChD	Electrochemical detector
EMIT	Enzyme Multiplied Immunoassay Technique
Enzyme	B-glucuronidase
Et_2O	Ether
EtOAc	Ethylacetate
EtOH	Ethanol
FBB	Fast Blue B
FBB-RR	Fast Blue B-RR
FE	Faeces
FL	Flourescence
FID	Flame ionisation detector
GC	Gas Chromatography
GC CIMS	GCMS with chemical ionisation
GCEIMS	GCMS with electron impact

GCMS	Gas Chromatography - Mass Spectrometry
Hex	Hexane
HPLC	High Performance Liquid Chromatography
iAmyOH	Isoamylalcohol
iPrOH	Isopropyl alcohol
MeCN	Acetonitrile
MeI	Methyl iodide
MeOH	Methanol
MIM	Metastable ion monitoring
MS	Mass Spectrometry
NA	Not Available
NH_3	Ammonia
Na_2SO_4	Sodium Sulphate
OH^-	Base-catalysed hydrolysis
PC	Paper Chromatography
PFBB	Pentafluorobenzoyl bromide
PFPA	Pentafluoro propionic anhydride
PFPOH	Pentafluoro propanol
PL	Plasma
RC	Radiochemistry
Rgt.	Reagent
RIA	Radioimmunoassay
SIM	Selected ion monitoring
SL	Saliva
std	Standard compound
TFAA	Trifluoroacetic anhydride
THC	^{9} - Tetrahydrocannabinol

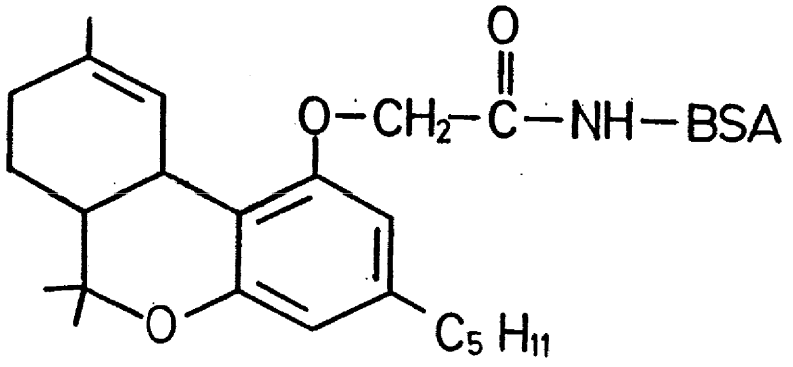
THC-COOH	11-nor-THC-9-COOH
THC-OH	THC-11-OH
TLC	Thin Layer Chromatography
TMAH	Tetramethyl ammonium hydroxide
TMCS	Trimethylchlorosilane
TIS	Tissue
UR	Urine
vis	Visual
^{3}H	Tritium
*	References cited therein

2.1 Radio Immunoassay (RIA)

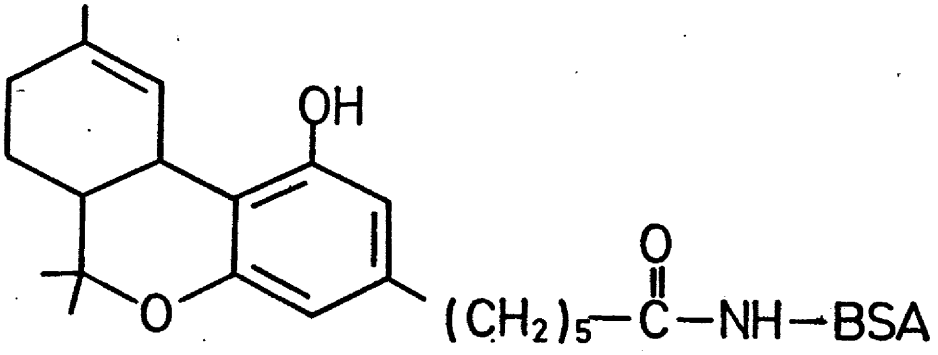
The principle of RIA is based on the competitive binding of antibody to radiolabelled and unlabelled antigen. The radiolabelled antigen - antibody complex is commercially available. The unlabelled antigen refers to the drug in the biological fluid that is to be quantitated. The radiolabelled antigen-antibody complex varies according to the type of drug that is to be detected. The chemical structure of the radiolabelled antigen should be similar to that of the drug. When the labelled antigen-antibody complex is added to a solution of the drug, labelled antigen is released when the antibody recomplexes with the drug. The free labelled antigen can be detected in a scintillation counter. The amount of released radiolabelled antigen thus reflects the quantity of drug present. Accurate quantitative data relating to drug concentration can thus be obtained.

Two typical antigens used in the analysis of cannabinoids (in particular THC) in body fluids are illustrated in Figure 8 (13, 14). The attachment of the protein bovine serum albumin (BSA) is necessary for the antibody complexation process.

The 3 types of radiolabels used in cannabinoid RIA's are ^{3}H, ^{14}C or ^{125}I. Although tritium (^{3}H) has a better shelf-life than ^{125}I, it is not as popular because of its lower specific activity[15]. ^{125}I however has a higher theoretical specific activity. This, together with its resistance to quenching and ease of measurement make it a suitable radioligand[16]. The



O-CARBOXYMETHYL-THC-BOVINE SERUM ALBUMIN
(CBM-THC-BSA)



5′-CARBOXY-THC-BOVINE SERUM ALBUMIN
(THC-CO-BSA)

FIGURE 8 **The Two Types of Antigens used in RIA**

drawback of ^{125}I is that its size makes introduction to small molecules difficult and may cause steric hindrance to antibody binding. In the case of THC this is overcome by substitution at the 5' position.

Because of the principle of RIA, there is cross-reactivity with other cannabinoids. Thus measurements are based on total cannabinoid levels as opposed to just THC or THC-COOH. This could be an advantage where only the presence of cannabinoids is the criteria, as in the determination of recent cannabis consumption.

The main advantages of RIA over some of the other analytical techniques are[17]:-

i. No pretreatment of biological fluid is necessary
ii. Sensitivity (0.25 to 5ng ml^{-1} cannabinoids)
iii. Simultaneous measurement of a large number of samples
iv. Selective antiserum depending on drug to be measured

The main disadvantages of RIA are:-

i. Lack of specificity
ii. Interference from substances in plasma (not in urine)
iii. Sensitivity to buffer condition
iv. Lacks precision because of the variables inherent in the preparation of reagents by serological procedures
v. Involves radioisotopes and therefore precautions against radioactive waste have to be taken

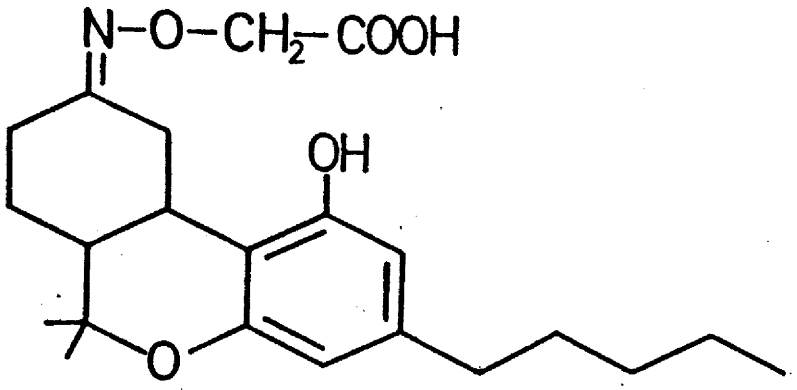
In order to improve the specificity of an RIA method, HPLC has been used to separate THC and THC-COOH from other cannabinoids that may be present in body fluids, prior to RIA analysis[9,17,18]. Using this method a detection limit of 0.1 ng ml^{-1} was achieved.

2.2 Enzyme Multiplied Immunoassay Technique (EMIT)

EMIT is a homogenous enzyme immunoassay that was first used for the measurement of morphine in urine. It employs an enzyme as label. The principle of EMIT is based on the activation of an enzyme, malate dehydrogenase (MDH), in the presence of the drug to be detected.

The enzyme is chemically attached to the hapten, which closely resembles the drug to be detected. The enzyme, although conjugated to the hapten, is active and capable of indirectly converting NAD to NADH. Antibodies against the hapten are produced separately and complexed with the enzyme-hapten conjugate. This enzyme-hapten-antibody complex is unable to convert NAD to NADH. The commercially available reagent mixture is added to a solution of the drug, the antibody detaches itself from the enzyme-hapten conjugate and complexes with the free drug. The enzyme conjugate is now activated and converts NAD to NADH. This enzymic reaction is monitored spectrophotometrically between two time intervals. The rate of the reaction depends on the amount of activated enzyme present, which in turn depends on the amount of drug present.

The antigen used in the EMIT cannabinoid assay, THC-MDH conjugate, is shown in Figure 9. The principle of the EMIT cannabinoid assay is illustrated in Figure 10. During the measurement, the reaction is monitored at 340nm and the absorbance difference measured between T=15 sec and T=45 sec is printed.



THC-MDH CONJUGATE

FIGURE 9 **The Antigen used in the EMIT Cannabinoid Assay**

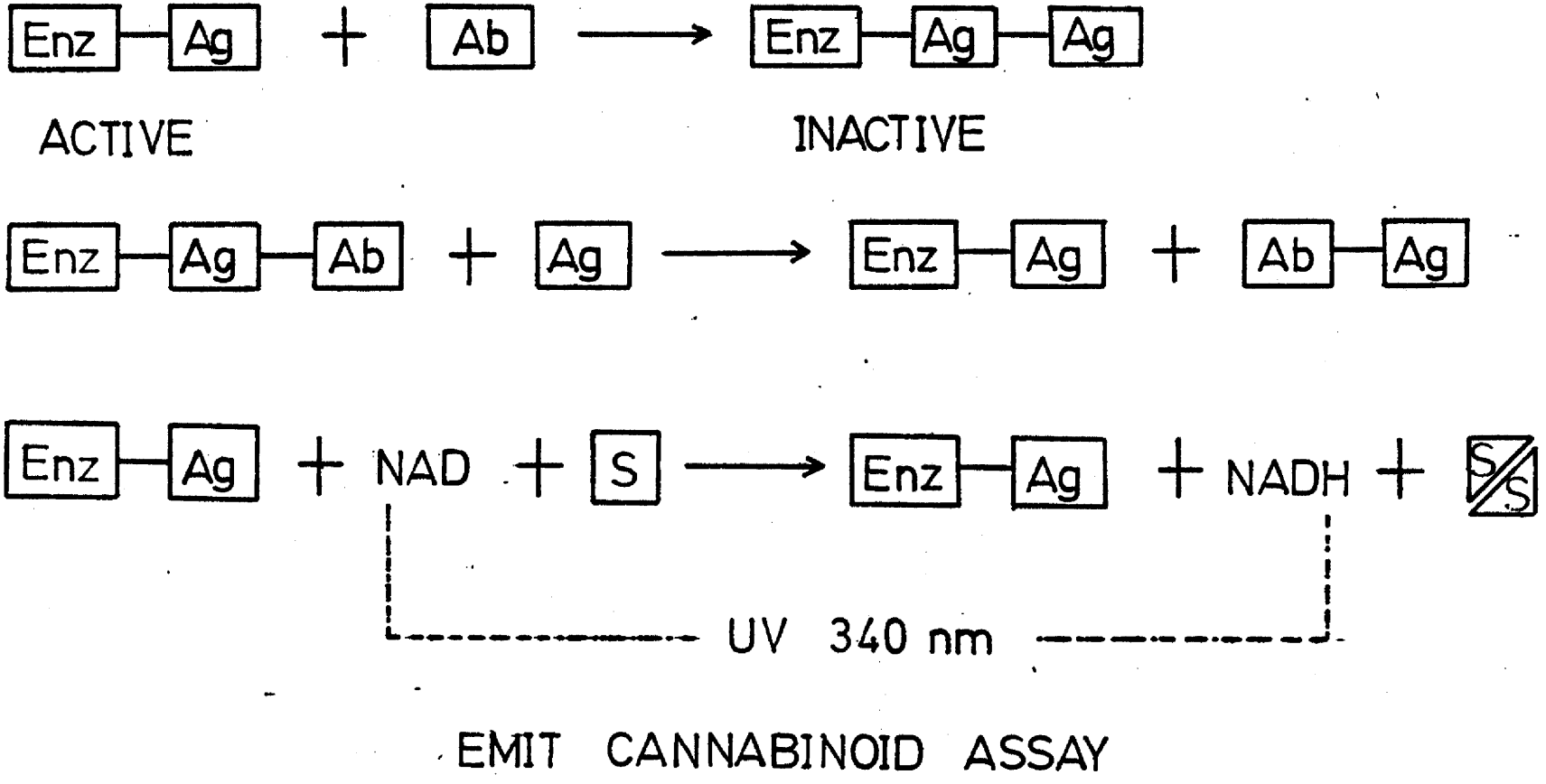

Enz
Ag
Ab
ACTIVE
INACTIVE
NAD
S
NADH
UV 340 nm
EMIT CANNABINOID ASSAY

FIGURE 10

This Δ A value is thus a measure of the amount of drug in the sample.

The EMIT cannabinoid assay is designed to test urine rather than blood (plasma) samples. This is an advantage for laboratories wishing to use non-invasive techniques to obtain samples, or where there are no personnel trained to obtain blood samples[19].

The assay is designed to be most sensitive to THC-COOH and THC-OH. It will also detect other cannabinoids such as THC, but with reduced sensitivity.

The EMIT cannabinoid kit contains 3 calibrators which are used in the assay. The 3 calibrators are lyophilised urine samples, which when reconstituted vary in their concentration of THC-COOH as follows:

Negative calibrator	-	0ng ml^{-1}
Low calibrator	-	20ng ml^{-1}
Medium calibrator	-	75ng ml^{-1}

Each kit contains sufficient reagents to run 100 samples.

The Low Calibrator is called the "cut-off" calibrator, as samples producing a response (ΔA value) equal to or greater than that of the Low Calibrator are interpreted as positive. Samples that produce a response less than that of the Low Calibrator are interpreted as negative. It can be used to iden-

tify positive samples that contain as little as 20ng ml^{-1} of THC-COOH (and THC-OH). The manufacturers state that 95% of the time positive or negative interpretation will be correctly made for samples containing 50ng ml^{-1} or 0ng ml^{-1} THC-COOH, respectively. For samples containing more than 50ng ml^{-1} of the cannabinoid metabolite, the correct interpretation will be made for correspondingly more than 95% of the samples.

The calibrator responses can also be used to obtain a semi-quantitative interpretation for a sample. This is carried out by plotting the calibrator responses against log concentrations. The resulting calibration curve can be used to evaluate samples (Figure 11).

The main advantages of EMIT over the other analytical techniques are:-

i. absence of sample pre-treatment
(EMIT is a homogenous enzyme immunoassay)

ii. small sample volume required

iii. ease of sample collection (urine vs plasma)

iv. short analysis time (1 minute)

v. semi-quantitative data obtainable with comparative ease

vi. the EMIT procedure does not require skill in execution

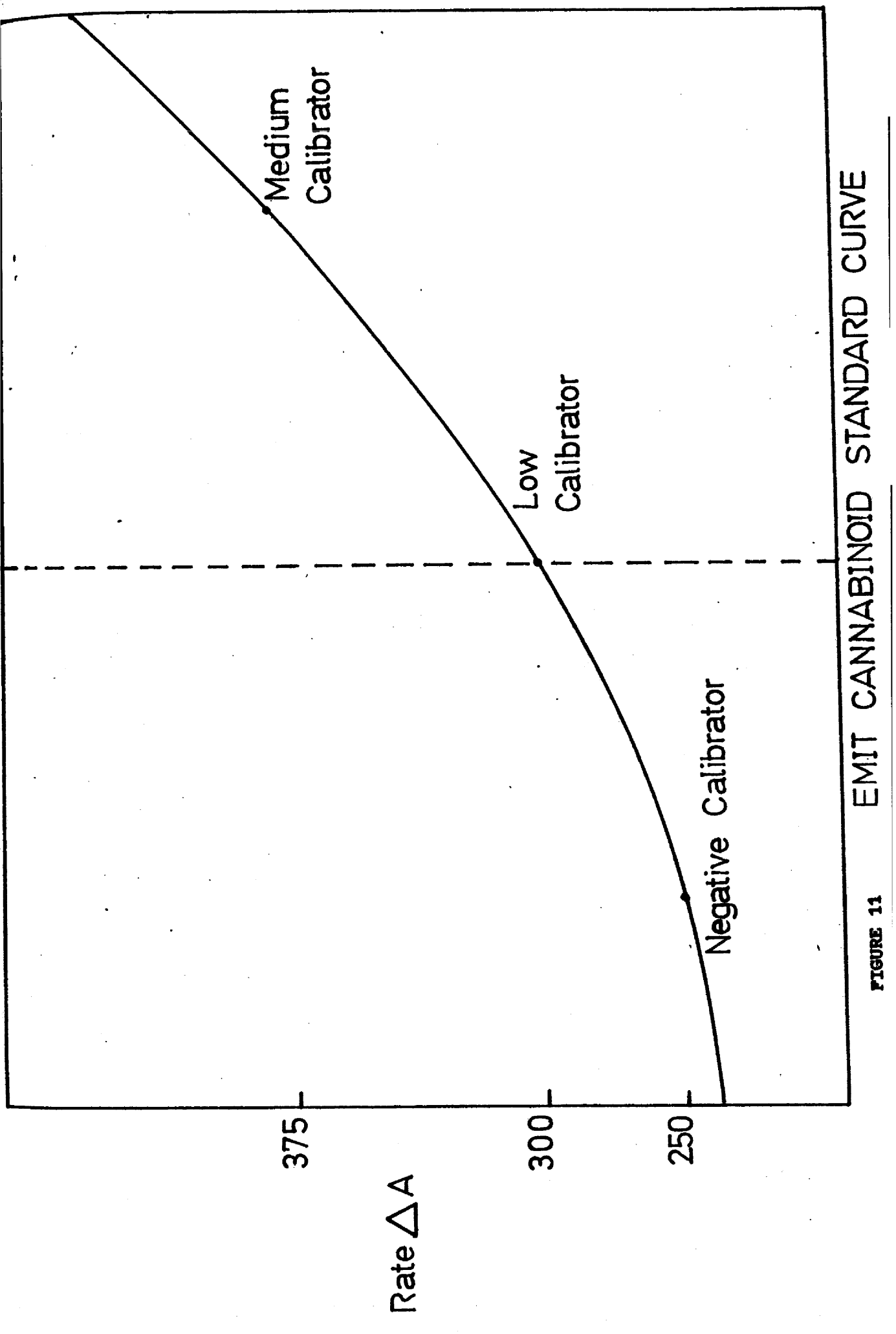

EMIT CANNABINOID STANDARD CURVE
Rate ΔA
375
300
250
Medium Calibrator
Low Calibrator
Negative Calibrator

FIGURE 11

2.3 EMIT vs Other Methods

The reliability and accuracy of the EMIT cannabinoid assay method has been questioned and a comparisons have been made with other more established techniques.

In one such comparative study between EMIT and a tritiated RIA kit, the latter was found to be a very difficult and time-consuming procedure. The study recommended that the RIA kit should not be used by inexperienced personnel for quantitation purposes. Furthermore, since this RIA kit was geared for the detection of THC and not THC-COOH, it was questionable as to its applicability in forensic analysis where the expected concentrations of THC are comparatively low. This doubt arose even though the RIA was more sensitive (5ng ml^{-1}) than EMIT. In this study the authors recommended the development of an iodinated (I^{125}) tracer for both THC and THC-COOH. Such an RIA procedure might, in their opinion, overcome some of the problems encountered with the ^{3}H RIA procedure[11].

In another RIA vs EMIT study, good correlation existed between the two methods. Of the 37 samples tested, one sample gave a positive by EMIT but a negative by RIA. The EMIT response of this sample was just above the EMIT cut-off level and this could account for the false positive reading. The authors also considered the EMIT 20ng ml^{-1} cut-off point to be suitable if a high incidence of false positives is to be avoided. However, because of the very much lower cut-off limit of the RIA (1ng ml^{-1}), the incidence of false negatives by the EMIT was relatively high (25%)[20].

In the same study, good quantitative correlation was obtained between RIA and HPLC, and between EMIT and HPLC, for THC-COOH in urine.

The authors, however had certain misgivings regarding the use of EMIT. They found that the performance of the EMIT assay was affected by factors not previously encountered with other EMIT assays. The main problem was a drift (of the spectrophotometer absorbance reading) in the response from a given standard over a period of as little as 20 minutes (equivalent to 12 analyses). This meant that the calibrators had to be re-run periodically and resulted in the reduction of the number of effective tests per kit. Another problem encountered was the difficulty in obtaining duplicate A within 6 units as recommended by the manufacturer. In about 30% of the samples, a third replicate determination was required. This problem could be associated with the earlier one of a drift in the spectrophotometer readings.

Another factor working against the EMIT is the higher cost per sample compared to the RIA.

The authors noted that for the analysis of small numbers of samples (less than 10) the EMIT assay is easier to set up and gives faster results. For larger number of samples, the RIA method is less demanding on the operator, although the operator times are comparable. It is however possible to upgrade the EMIT system to make it fully automated, capable of processing up to sixty samples per hour.

The same authors have more recently developed a ^{125}I RIA method for the analysis of THC and its metabolites in body fluids[15]. This method was found to be in good quantitative agreement with EMIT. The authors considered the RIA method to be superior to the EMIT procedure in terms of:

i. ease of operation
ii. reliability
iii. sensitivity (cut-off = 2 ng ml^{-1})
iv. cost (1/10 to 1/100 the cost of comparable EMIT reagents)
v. convenience
vi. speed
vii. demand on the operator
viii. large scale screening

A comparison with a TLC method (for THC-COOH) has shown a good correlation with EMIT for samples at 0 ng ml^{-1} or above 50 ng ml^{-1}. However, for samples between 0 and 50 ng ml^{-1} the correlation was poor for EMIT. One possible reason for this poor correlation could be that whereas EMIT measures more than one cannabinoid, whereas TLC only measures THC-COOH[21].

In another study where EMIT was compared with TLC, good qualitative correlation existed. In this study, the hydrolysed urine was purified by passage through a Bond Elut - THC column prior to TLC. The sensitivity of this method (20 ng ml^{-1}) was comparable to EMIT and the authors recommended that this method be used as a confirmatory method for the EMIT cannabinoid screening procedure. However this TLC method requires 20 ml of urine[22].

In yet another study comparing a TLC method with EMIT, the authors proposed that the TLC procedure be used for mass testing

and only the positive urines be confirmed by EMIT. This was based on the 100% correlation between the two methods. The TLC method was considered rapid and cheap[23].

A study comparing EMIT with GC and GCMS shows 100% correlation for 29 samples with an EMIT response below the Low Calibrator level (20 ng ml^{-1}). All 6 samples between the Low and Medium (75 ng ml^{-1}) Calibrator responses were negative by GC and GCMS. This zero correlation could be due to the fact that all the samples had EMIT responses very close to the lower cut-off point and could therefore be devoid of cannabinoids. Of the remaining 27 samples which produced EMIT values greater than 75 ng ml^{-1}, confirmation was established by GC and GCMS for 22 of them. The remaining 5 were negative by both procedures. The authors concluded that although GC is not as sensitive as GCMS, it can be used as a confirmatory method for THC-COOH in urine which produce EMIT responses greater than 75 ng ml^{-1}[24].

EMIT has also been adapted for the detection (screening) of cannabinoids in blood. The cut-off point was set at 40 ng ml^{-1}, although THC-COOH was detectable down to 10 ng ml^{-1}. All samples found positive by EMIT were confirmed by GCMS. No cross-reactivity was found with other commonly misused drugs. During the EMIT procedure, the time interval between the two absorbance readings was increased to 80 seconds[25].

2.4 Extraction Procedures

Unlike the above mentioned immunological procedures, the other group of analytical methods (chromatography) require the drug sample (urine or blood) to be pretreated. The main pretreatment procedure common to all is extraction.

Apart from being essential, an extraction procedure should be effective. The main criteria for any extraction process is:-

i. speed

ii. quantitative transfer of drug

iii. suitability for any subsequent steps

Over 80% of the excreted cannabinoid metabolite THC-COOH is in the form of the glucuronide conjugate. The conjugate is very hydrophilic and polar and therefore not easily extractable from body fluids nor suitable for chromatography per se. Cleavage of the glucuronide ester linkage releasing the free acid would overcome the above drawbacks. This cleavage is achieved by hydrolysis.

Although hydrolysis is usually carried out on the urine or plasma sample prior to extraction, some published analytical procedures for the detection of THC-COOH proceed without this step[26,27].

The two main types of hydrolyses performed are base-catalysed or enzymic. Base-catalysed hydrolysis is considered more efficient and reproducible than either acid-catalysed or

enzymic hydrolyses[28]. In enzymic hydrolysis the enzyme employed is β-glucuronidase[21,29,30]. (see table 1).

In some instances, prior to extraction, the hydrolysed urine is partially purified by microcolumn chromatography. It is usually either Bond-elut[22,28] or Sephadex LH-20[12,26,31,32]. Sephadex LH-20 removes interfering lipids and metabolites usually present in plasma extracts. Bond-elut is a bonded phase silica gel column commonly used for sample clean-up.

Extraction of the THC-COOH in hydrolysed urine occurs at acidic pH, which ensures that the metabolite is neutral and thus soluble in organic solvents. The commonly used solvents and solvent mixtures for cannabinoid extraction that have been published are petroleum ether, hexane, ether, chloroform, combinations of petroleum ether and hexane or hexane and ethyacetate (See Tab e 1). Petroleum ether or hexane are favoured for extraction from plasma whilst hexane-ethyl acetate mixtures are common with urine extraction. In one study[17] the hydrolysed urine was mixed with a buffer-methanol mixture and analysed directly by HPLC.

The urine extract can then be analysed by TLC and HPLC, or derivatised for analysis by GC and GCMS.

2.5 Thin Layer Chromatography (TLC)

TLC has been used on various occasions for the detection of cannabinoid metabolites in body fluids. It has been used as a screening method or sometimes as a confirmatory test. TLC has also been applied to tissue[33], and plasma[9,10,21,22,34,37].

The stationary phase used in all the TLC methods was silica gel. Visualisation of the developed chromatogram was either by fluorescence[9,34,35,36] or by using a spray reagent.[10,21,22,37].

One of the fluorescent derivatives used for detecting cannabinoids is 2-p-chlorosulphonyl-3-phenylinone, which is claimed to be as sensitive as GC and GCMS, using 5ml of urine[36]. A spray reagent (sodium methoxide) is used in conjunction with the fluorescent derivative, to give a green fluorescence. The detection limit is 0.1 ng and samples with a cannabinoid concentration of 0.4 ng ml^{-1} can be detected easily. It is suggested to be ideally suited for routine screening. The derivative is unstable and measurement has to be made within a few minutes of TLC.

Another fluorescent derivative in use is dansyl chloride (5-dimethylaminonaphthalene-1-sulphonyl chloride), which has been used for the detection of cannabinoids in saliva[35]. Detection limits in the picomole (subnanogram) range are claimed. The TLC results were confirmed by mass spectrometry. The TLC procedure was sensitive enough to detect THC in saliva up to two hours after smoking. Another related flourescent

derivative, dabsyl chloride (4-dimethylaminoazobenzene-4'-sulphonyl chloride) has also been used with plasma and urine extracts, yielding a detection limit of 1 ng[34].

A combined radiolabelled fluorescent technique using ^{14}C dansyl chloride has also been tried out, yielding a sensitivity of a few ng ml^{-1} of THC in plasma[9]. Radiolabelled TLC, however, is not popular due to inaccuracies in measurement[12].

Using a spray reagent such as Fast Blue B (FBB) or Fast Blue B RR (FBB-RR) on the developed TLC plate is by far the most convenient and popular method of cannabinoid detection. A pink or red colouration is obtained for THC-COOH[10,21,22,37]. FBB-RR (4-benzoylamino-2,5-diethoxybenzene diazonium chloride) is considered to be more sensitive and gives a better colouration than FBB (di-O-anisidine tetrazolium chloride)[38]. With FBB, the colour fades quite easily at low cannabinoid concentrations[33]. Furthermore, FBB is considered carcinogenic whereas FBB-RR is not[22]. In addition, FBB cross-reacts with 3 non-cannabinoid herbs rosemary, sage and thyme[21]. This cross-reactivity is a drawback especially in temperate regions where these herbs are more readily available. These spray reagents are not as sensitive as the fluorescent derivatives, and provide detection limits in the 20-50 ng ml^{-1} (urine) range[10,22].

To preserve a TLC chromatogram, diethylamine should be sprayed before and after the application of the spray reagent[38].

Quantitative work can be performed by eluting the sprayed component from the TLC plate and measuring by UV. It should be noted that the intensity of the colour is dependant on pH, temperature and time. Alternatively densitometry may be used, provided the plate is sprayed with light mineral oil first to reduce background interference[33].

Different TLC solvent systems (mobile phase) have been used for the detection of THC-COOH[10,21,22,37]. In some instances the cannabinoid metabolite is derivatised prior to TLC[34]. Two-dimensional TLC has also been used[21].

TLC has been considered a non-screening procedure in conjunction with EMIT[23] and MS[37]. Conversely, it has been suggested that TLC be used as a confirmatory test after EMIT[22].

2.6 High Performance Liquid Chromatography (HPLC)

Although HPLC is becoming increasingly important for its application to trace analysis of drugs and metabolites, its use in the detection of cannabinoids in biological fluids has been somewhat restricted. This is because the commonly used UV (ultraviolet) and RI (refractive index) detectors are not sensitive enough[39]. Thus some other detection method is subsequently used after HPLC, such as RIA, GC, GCMS or MS. In such instances HPLC is used as a purifying technique. HPLC is performed in the reverse-phase mode.

The most widely used combination has been HPLC-RIA, which combines a good separation technique with a sensitive detection procedure[9,17,18]. Sensitivity levels down to 1 ng ml^{-1} are possible. This technique has been used for the quantitation of cannabinoids in plasma and urine.

HPLC followed by GC detection has been tested on THC[40] and THC-COOH[30]. This combination is suggested to be suitable and effective. Both qualitative and quantitative results obtained were verified by GCMS. The sensitivity of the HPLC-GC procedure was 20 ng ml^{-1} (THC-COOH). The HPLC (reverse phase) was monitored by UV and the eluate collected, extracted, derivatised and injected into the GC. The GC was equipped with an ECD detector[30].

HPLC in conjunction with MS has been used for the detection of THC in plasma[41]. Various human body fluids and tissue were used.

The detection of THC and THC-OH from plasma has been carried out using HPLC with an electrochemical detector. This method is able to detect cannabinoids down to 5 ng ml^{-1}. The electrochemical detector is sensitive and fairly selective in that only compounds containing an electrochemically oxidisable or reducible functional group can be detected[39].

HPLC has also been recently used with just a UV detector for THC-COOH in urine. The sensitivity of the proposed method was 25 ng ml^{-1}. Here, the hydrolysed urine was purified using Bond-Elut columns prior to HPLC[28].

2.7 Derivatisation Procedures

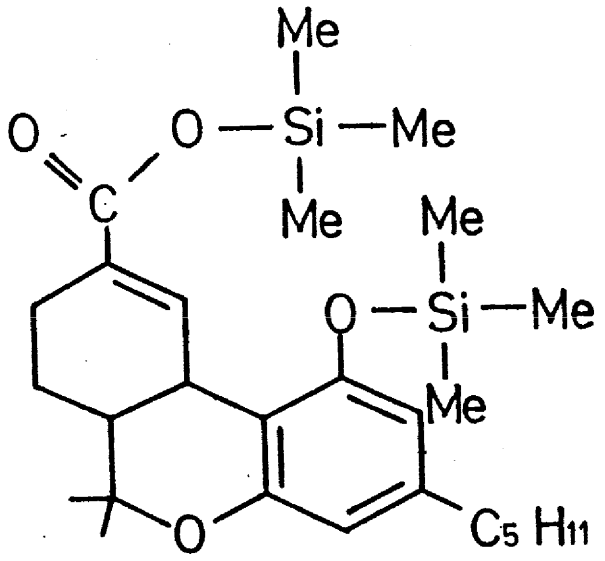
The presence of a phenolic hydroxyl and in particular, a carboxyl functional group in THC-COOH, necessitates their derivatisation before GC or GCMS can be carried out. Underivatised THC-COOH would be strongly adsorbed onto the stationary phase of the GC (GCMS) column and not be eluted, or eluted very slowly.

Another function of the derivatisation process is to enhance the detectability of the drug, when an appropriate detector is used. For instance in GC fluorinated derivatives enhance the sensitivity using an electron-capture detector (ECD). The electronegative fluorine atoms are suitable for electron capture, the basis of the ECD.

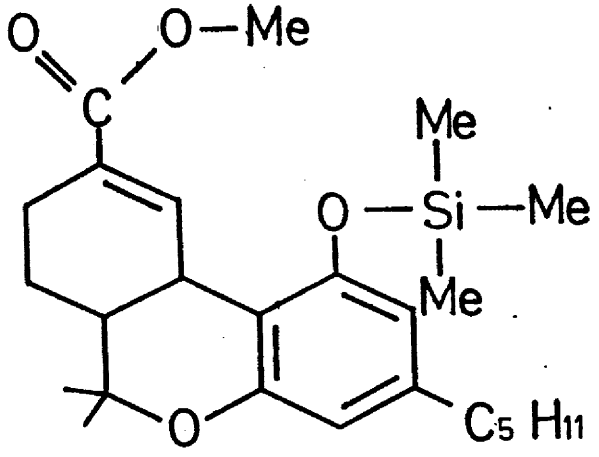
For normal GC-FID or GCMS work, silyl derivatives (Figure 12) are usually prepared. The most popular silylating agents are Bis-trimetlylsilylacetamide (BSA)[13,33] and Bis-trimethylsilyl trifluoroacetamide (BSTFA)[9,12,29,32]. Generally, silylation of acidic cannabinoids occurs much more rapidly at elevated temperatures (e.g. 100°C for 30 minutes).

Sometimes two derivatising reactions are undertaken in succession, using diazomethane (CH_2N_2) and BSA. Diazomethane methylates the carboxyl function to give the methyl ester. BSA then silylates the phenolic hydroxyl to give the methyl ester trimethylsilyl ether of THC-COOH (Figure 12)[26,32].

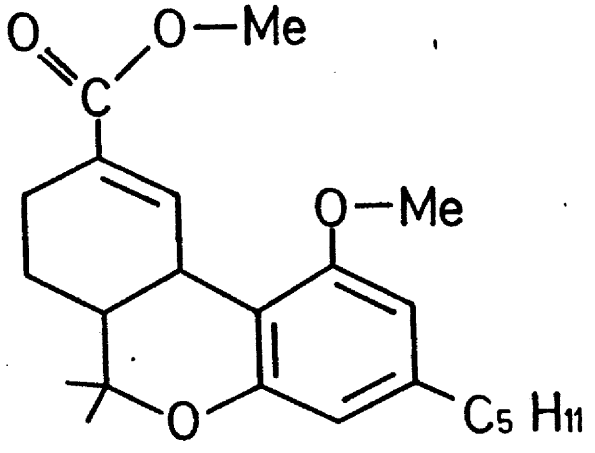
For fluorinated derivatives (for GC-ECD) the main derivatising agents used are pentafluorobenzylbromide (PFBB),[40,43]



TMS-ESTER TMS-ETHER OF THC-COOH



METHYL-ESTER TMS-ETHER OF THC-COOH



METHYLESTER METHYLETHER OF THC-COOH

FIGURE 12 **The Main Derivatives of THC-COOH**

pentafluoropropionic anhydride (PFPA)[12,30,42] and trifluoroacetic anhydride (TFAA)[27].

Methylation of THC-COOH to give the methyl ester methyl ether has also been employed. The methylating agent used is methyliodide (CH_3I) in base (Figure 12)[24,37].

2.8 Gas Chromatography (GC)

Detection of cannabinoids by GC is a popular and sensitive method. The sensitivity is enhanced with an electron capture detector (ECD) which is capable of determining low levels of cannabinoids encountered in biological fluids.

Most of the reported GC procedures utilise the ECD for detecting THC[40,42], THC-OH[12] and THC-COOH[30,43] from biological extracts. The sensitivity of the GC-ECD procedures is about 1 ng ml^{-1} (see Table 1) in urine or plasma. One of the GC-ECD procedures developed recently for THC-COOH in urine was considered suitable because it was sensitive, reproducible, required a shorter analysis time, minimum sample preparation and needed only 1ml of urine.

A GC-FID procedure has been developed for the detection of THC-COOH in urine, with a sensitivity level of 20 ng ml^{-1} which was considered suitable as a confirmatory method to EMIT.

The commonly used stationary phases for GC are the methyl silicones (eg. OV-101 and SE-30) and the more polar methyl phenylsilicones (eg. OV-17). The retention times observed are usually under 10 minutes, depending on column type and derivative. Packed column chromatography is currently the main method used in the GC of cannabinoids. Capillary GC, in conjunction with MS has recently been used, with improved sensitivity[27].

A dual-column dual-oven GC instrument has been used with

an ECD detector for the detection of THC in plasma. The first column acts as a purifier by removing interfering substances in the extract. Levels down to 0.5 ng ml^{-1} could be detected[42].

Negative ion GCCIMS is more sensitive and more selective than positive ion GCCIMS, as evidenced by the detection limit of 0.1 ng ml^{-1} of THC-COOH in plasma or urine. Detection levels of 0.2 and 0.5 ng ml^{-1} were obtained for THC and THC-OH, respectively[27]. In this procedure, trifluoroacetic anhydride (TFAA) was used as the derivatising agent. The introduction of trifluoroacetyl groups into the cannabinoid structure increased the electron affinity of the compound. Another reason for the high sensitivity is the use of a capillary column instead of the usual packed column.

The earlier methods, utilising GCEIMS gave senstivities in the region of 1-5 ng ml^{-1}. The sensitivities may be enhanced by operating the MS in the selected ion monitoring (SIM) mode, where the detector is tuned to pick up a selected number of ions only[32].

A technique known as probability based matching (PBM) has been used in the GCMS of cannabinoids. PBM yields quantitative information and also provide an estimate of the degree of specificity of the particular procedure used[29].

GCMS is the most suitable confirmatory method chosen, when a doubt arises in one of the other chromatographic methods eg. interfering peaks in GC and HPLC, or a cross-reacting substance in EMIT.

2.9 Gas Chromatography-Mass Spectrometry (GCMS)

GCMS is a very powerful tool in the detection and identification of drugs and metabolites. It is a combination of a good separation method (GC) and a sensitive detector (MS) which is capable of detecting and positively identifying an eluted component. The MS does the latter by producing a mass fragmentation pattern and a molecular ion.

The two main methods of sample ionisation in MS (GCMS) are electron impact (EIMS) and chemical ionisation (CIMS). EIMS is a high energy ionisation process which results in a strong fragmentation pattern at the expense of a weak molecular ion (M^+). CIMS, however is a "soft" ionisation process which produce an intense M^+ and a weak fragmentation. For drug detection purposes, CIMS is favoured over EIMS because a drug is usually identified by its molecular ion. In structural chemistry, however, where the structure of an organic compound needs to be elucidated, EIMS is the popular method as the fragmentation pattern is dependent on structure.

CIMS requires a reagent gas for ionisation (protonation) to occur. This gas may be either ammonia, methane or isobutane. In addition to positive ion CIMS, there is also negative ion CIMS.

In cannabinoid detection procedure using GCMS, the main types of GCMS used have been:-

i. GC EIMS[12,26,27,29,31,33,37,41,45]

ii. GC CIMS - ammonia reagent gas[9,32])
- methane reagent gas[30,44]) positive
- isobutane reagent gas[12]) ion

iii. GC CIMS - negative ion[27]

In cannabinoid analysis, GCCIMS using ammonia as reagent gas is considered more sensitive and more specific than either GCEIMS or GCCIMS using either methane or isobutane as reagent gas[11]. The disadvantage of ammonia reagent gas is that no fragment ions are formed. Methane, however, does produce limited fragmentation[44]. Sometimes fragmentation is useful in confirming the identity of a particular compound.

CHAPTER 3

AN OVERVIEW OF THE EXPERIMENTAL DESIGN

In the previous chapter the various analytical methods for the detection of cannabinoids in body fluids were discussed. It was observed that the sensitivity, speed and reliability of the different methods varied. The same variation applies to the different procedures that have been used within each method.

The remainder of this report will focus on the development of suitable procedures for the detection of the principal cannabinoid, THC-COOH, in urine. Procedures were developed for TLC, GC and GCMS. The results of authentic samples using these methods were used to compare with those obtained by EMIT.

Before any of the 3 chromatogrphic procedures could be developed, a suitable extraction method had to be established for THC-COOH in urine. This extraction method was subsequently used in conjunction with all the chromatographic procedures. It was also used for the bulk extraction of THC-COOH from urine. This bulk extract served as a pseudo-standard during the initial development stages of TLC, GC and GCMS. The bulk extracted THC-COOH was "cleaned up" by preparative TLC prior to use.

The development of the chromatographic procedures was carried out systematically - TLC followed by GC. The conditions for GC analysis were then transferred to GCMS, with slight modifications. The

sensitivity limits and minimum sample volume required for each procedure were estimated.

Finally, using the same urine samples the EMIT procedure was checked against the chromatographic methods for its reliability. The cannabinoid concentration of the urine samples were initially estimated semi-quantitatively by EMIT.

Losses during the extraction procedure and the derivatisation step were estimated using EMIT and by data from GC.

Cross-reactivity studies using a selection of commonly abused drugs were carried out on the EMIT. Preliminary work was also undertaken to determine the stability of cannabinoids (THC-COOH) in urine under different storage conditions.

CHAPTER 4

EXTRACTION PROCEDURES

A suitable extraction procedure is necessary to ensure that the THC-COOH is extracted quantitatively and as selectively as possible. A quantitative recovery is important when only small quantities of the sample (i.e. low urine volume) is available, thus reducing the total amount of cannabinoids present. A selective extraction procedure ensures that interfering substances present in the urine are removed.

For a quantitative and selective extraction, a suitable extraction solvent should be used. Additionally, the solvent should be volatile to ensure ease of handling for subsequent analysis.

A suitable extraction procedure developed here for THC-COOH in urine is presented in the following pages of this chapter.

4.1 Materials

Ether, cyclcohexane and ethylacetate (all BDH) were of AR grade and not purified further. Potassium hydroxide (BDH) was of GPR grade. Universal pH indicator strips were obtained from Merck. A vortex mixer from Griffin and George was used for mixing.

4.2 Method

The procedure for the extraction of THC-COOH from urine can be presented in a stepwise manner, where each step is designated a particular function (boxed in), as shown below:-

1.	Take 3 ml of urine and add 0.3 ml 10 N KOH. Vortex the mixture for 2 seconds. Cap tightly.	Basification
2.	Place in an oven or water bath at 50°C for 30 minutes.	Hydrolysis
3.	On cooling, add 3ml of ether and vortex the mixture for 2 minutes. Centrifuge and discard the ether layer.	Purification
4.	Add 0.3 ul 1M KH_2PO_4 followed by a few drops of 50% HCL until the urine reaches pH1-2. Vortex for 2 seconds.	Acidification
5.	Add 3ml of cyclohexane - ethyl acetate (96:4). Vortex for 2 minutes. Centrifuge and discard the lower aqueous layer.	Extraction
6.	Add 3ml 0.2 N NaOH and vortex for 2 minutes. Discard the upper organic layer.	Back Extraction
7.	Add a few drops of 50% HCl until the pH falls to between 1 and 2. Vortex for 2 seconds.	Re-acidification
8.	Add 3ml of cyclohexane-ethyl acetate (96:4). Vortex for 2 minutes. Discard the lower aqueous layer.	Re-extraction
9.	Evaporate the organic phase to dryness under a stream of nitrogen at a slightly elevated temperature (50°C).	Concentration

4.3 Discussion

An explanation of each of the steps involved in the extraction procedure will be presented in this section. It should be noted that all glass-ware used in the procedure was silylated beforehand by rinsing in 2% dimethyl chlorosilane (DMCS) in toluene and heating in the oven until dry. The purpose of silylation is to ensure that irreversible adsorption of cannabinoids onto the surface of the glassware is reduced to a minimum.

The extraction procedure presented here is the result of modifications made to existing methods and the incorporation of some of the individual steps of these methods into the procedure reported here.

BASIFICATION: Studies have shown that base-catalysed hydrolysis of urine samples is more effective than either acid or enzymic hydrolysis for the release of unconjugated THC-COOH.

HYDROLYSIS: The glucuronide ester linkage in the conjugated THC-COOH is easily hydrolysable and so mild hydrolytic conditions, as used here, are sufficient. Higher temperatures for prolonged periods of time may have the detrimental effect of degradation of the cannabinoid metabolite. Higher temperatures (100°C) for a shorter length of time (10 minutes) have been reported to be satisfactory.

PURIFICATION: This step helps remove some of the basic and neutral substances from the urine which might otherwise get carried through the extraction procedure and interfere with

subsequent analysis. Ether is used at this stage because it has been shown here by TLC to be a suitable solvent for removing impurities in urine. The other solvents that were tested and found to be not as effective as ether were cyclohexane, hexane, chloroform, dichloromethane and various mixtures of hexane-ethyl acetate.

MODIFICATION: Acidification of the urine is necessary for the THC-COOH to be in its neutral state and therefore available for organic solvent extraction. The presence of the buffer (potassium dihydrogen orthophosphate, KH_2PO_4) helps to stabilise the acidic pH of the urine.

EXTRACTION: The solvent of choice in this laboratory for the extraction of THC-COOH is a mixture, cyclohexane-ethyl acetate (96:4). A comparison of the extracts of different solvents and solvent systems showed that the above mixture was the most suitable. This was because the relative amount of THC-COOH extracted was comparable to the other solvents tried. More importantly, the co-extraction of other substances (impurities) from the urine was minimal. These findings were based on the results obtained by running a TLC of the extracts and its visualisation by UV and spray reagent (Fast Blue B). A vortex time of 1½ - 2 minutes using a single volume of solvent is sufficient to transfer most of the metabolite into the organic phase.

BACK-EXTRACTION: This is a method of further purifying the

extract by selectively re-introducing the cannabinoid metabolite into the aqueous phase. The aqueous phase is alkaline, ensuring solubility of the ionised THC-COOH in the medium. Most of the impurities are left behind in the organic phase which is subsequently discarded.

REACIDIFICATION and REEXTRACTION: cf "ACIDIFICATION" and "EXTRACTION" above.

CONCENTRATION: This step is merely an evaporation step to remove the volatile organic solvent containing THC-COOH and to prepare the extract residue for subsequent analyses such as TLC or GC.

If the dried residue is not to be used immediately then it should be sealed and stored in the fridge until required. Storage in a freezer would be preferable if its going to be for any length of time. Acetone is a suitable solvent for redissolving the residue.

Another precaution that should be taken is to avoid direct sunlight at any time during the extraction procedure. Sunlight (UV radiation) might adversely affect the stability of the cannabinoid in urine.

Urine samples that are received for analysis should be stored frozen until required. Any remaining urine sample may be refrozen and kept if necessary, for future analysis.

Although the above procedure is a tried and tested one, modifications and refinements are currently being carried out at the dif-

ferent stages along the process. It is hoped that the duration of the extraction procedure may be shortened without affecting the efficiency of the process.

CHAPTER 5

THIN LAYER CHROMATOGRAPHY

TLC is the most popular of the chromatographic techniques in drug analysis, by virtue of its low cost, short analysis time, relative sensitivity and ease of operation. However, its use in the detection of cannabinoids in body fluids has been somewhat restricted due to the inherently low levels of the drug that have to be analysed. However, TLC has previously been used in cannabinoid analysis, and Table 2 provides a list of the reported solvent systems.

5.1 Materials

Aluminium backed pre-coated TLC plates from Merck were used throughout. The plates were coated with silica gel $60GF_{254}$ to a thickness of 0.2mm. The solvents used were of GPR grade and not purified further. Fast Blue B was purchased from BDH. TLC tanks and spray guns were purchased from Shandon Products.

5.2 Method

The residue extract to be analysed is dissolved in approximately 50ul of acetone. The solution is vortexed for a few seconds to ensure complete dissolution. A few microlitres of the solution is drawn up a capillary tube and repeatedly spotted onto a suitably marked TLC plate of approximate dimensions 3cm x 10cm. In between each spot application, a stream of cool air is passed over the plate to evaporate the solvent. Once dry, the spotting can be repeated. When the sample

application is complete, the plate is immersed in a pre-equilibrated TLC tank containing the mobile phase solvent ($CHCl_3$:MeOH:NH_3; 70:30:2). When the solvent front has travelled a distance of about 7 cm, the plate is removed from the tank and air dried.

The developed TLC chromatogram is sprayed with Fast Blue B spray reagent and air dried. The spray reagent is prepared by dissolving 40 mg of Fast Blue B in 40ml of MeOH:H_2O (3:1). The reagent should be prepared fresh daily, and stored at 0°C when not in use. THC-COOH appears as a pink spot at Rf 0.31.

5.3 Discussion

All the solvent systems listed in Table 2 were tested using the neutral cannabinoids (THC,CBN and CBD) and THC-COOH (prepared by bulk extraction of cannabinoid - containing urine). The most suitable system was $CHCl_3$: MeOH : NH_3 (70:30:2), which provided a good separation between THC-COOH and the rest of the cannabinoids. The Rf values observed were:

THC Rf 0.85
CBN Rf 0.85
CBD Rf 0.85
THC-COOH Rf 0.31

THC-COOH, at the levels used, is not particularly noticeable under either shortwave or longwave UV light. A spray reagent, Fast Blue B, was found to be sensitive enough to give a colour reaction with THC-COOH and other cannabinoids. For THC-COOH, a pink spot was observed a few seconds after spraying. The intensity of the spot developed fully a few minutes later. The spray reagent has to be

TABLE 2

TLC Solvent Systems Used in the Detection of Cannabinoids in Biological Fluids

REFERENCE	SOLVENT SYSTEM
33	$CHCl_3$: EtOH (4:1)
34	Hex: EtOAc: DEA (20:5:1)
35	Two dimensional: i. Hep: Acet (9:1); 1st direction ii. cycHex: Bz: MeOH: EtoAc (70:30:1:1); 2nd direction
	Two dimensional: i. Pet. ether: Et_2O: HOAc (40:10:1); 1st direction ii. Hep:Et_2O (4:1); 2nd direction
36	MeOH: H_2O (19:1)
21	Double development: i. Acet: $CHCl_3$: TEA (80:20:1); 1st development ii. Pet. ether: Et_2O: HOAC (50:50:1.5) 2nd development
22	EtOAc:MeOH:NH_3:H_2O (12:5:1:0.5)
10	$CHCl_3$:MeOH:NH_3 (85:15:2)
37	$CHCl_3$:MeOH:NH_3 (70:30:2)

freshly prepared before use for it to be fully effective, and it should be stored at 0°C when not in use. The sensitivity of the spray reagent decreases with storage. Whereas freshly prepared Fast Blue B solution is greenish, it turns pinkish on storage and loses its effectiveness.

Another spray reagent that has been used for detecting higher levels of cannabinoids is Beam's reagent. The cannabinoids produce a purple colouration on spraying. Beams reagent, which is 5% potassium hydroxide in ethanol, is relatively stable and can be stored at 4°C. However, due to its lack of sensitivity, it is not applicable in the analysis of cannabinoid metabolites.

The colouration produced on spraying will remain for a long time (a few weeks) if the TLC chromatogram is stored away from light.

The extraction and TLC procedure was used to analyse a batch of cannabinoid-containing urine samples. A semi-quantitative evaluation of the concentration of the cannabinoids in the urine samples was estimated using the EMIT technique and its three calibrators. The results showed that urine samples which gave an EMIT response greater than the Medium Calibrator (75 ng ml^{-1}) were positively identified as THC-COOH by TLC. This suggests that the TLC method can be used as a second method after EMIT, for samples giving an EMIT response greater than the Medium Calibrator.

CHAPTER 6

DERIVATISATION PROCEDURE

Derivatisation of THC-COOH is necessary to reduce the molecule's polarity and make it more suitable for analysis by GC and GCMS.

Various derivatising agents have been reported for use with THC-COOH. They have been mainly silylating agents alone or in combination with a methylating agent. Fluorinated derivatising agents are used in GC-ECD analysis.

In this laboratory an FID detector was used with the GC and so a normal silylating agent, BSTFA, was used for derivatising THC-COOH.

6.1 Materials

Bis-(trimethyl silyl)-trifluoroacetamide (BSTFA), was obtained from Supelco Co. (USA). Acetonitrile (Merck) was of analytical grade.

6.2 Methods

The residue obtained from urine extract was dissolved in 100ul acetone and transferred to a microvial (100ul, V-shaped) and the solution dried down under a stream of nitrogen. When the residue is totally dried (all traces of moisture have to be removed), 25ul of BSTFA (70% in acetonitrile) is added to the microvial and capped tightly. The microvial is vortexed for 30 seconds to ensure total dissolution, and placed in an oven at 90°C for 90 minutes.

On removal from the oven and subsequent cooling, the reaction solution is ready for injection into a GC. The normal volume injected is lul. If the reaction mixture is not used for analysis immediately, then it can be stored frozen until required.

6.3 Discussion

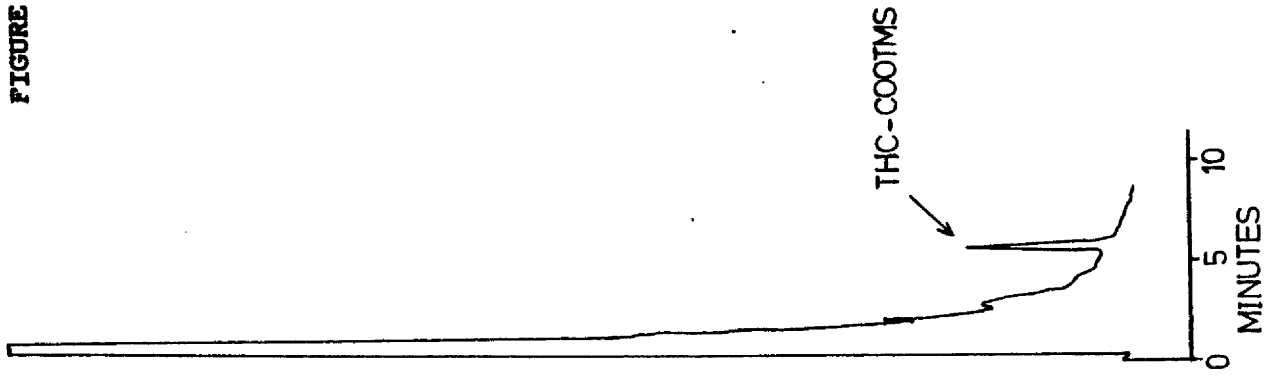
The choice of a fluorinated derivative for THC-COOH seemed unnecessary at this stage because although the GC in this laboratory had the capability of performing GC-ECD work, a GC-FID system was the more common method of analysis in local laboratories.

The normal derivatisation procedure for THC-COOH using GC-FID or GCMS can follow one of two main routes. The first would involve esterification of the carboxyl function using a methylating agent, followed by silylation of the phenolic group yielding the methyl ester trimethylsilyl ether of THC-COOH. The second popular route is the silylation of both functional groups to give the TMS ester TMS ether of THC-COOH.

In the first method (double derivatisation) silylation of the methyl ester of THC-COOH is fairly rapid and occurs at room temperature or at slightly elevated temperatures. In the second method (single derivatisation), although the silylation of the phenolic group is rapid, that of the carboxyl function is much slower and occurs at prolonged elevated temperatures.

In this laboratory the single derivatisation procedure was chosen from the point of view of convenience i.e. having only one deriva-

FIGURE 13 GC of THC-COOTMS



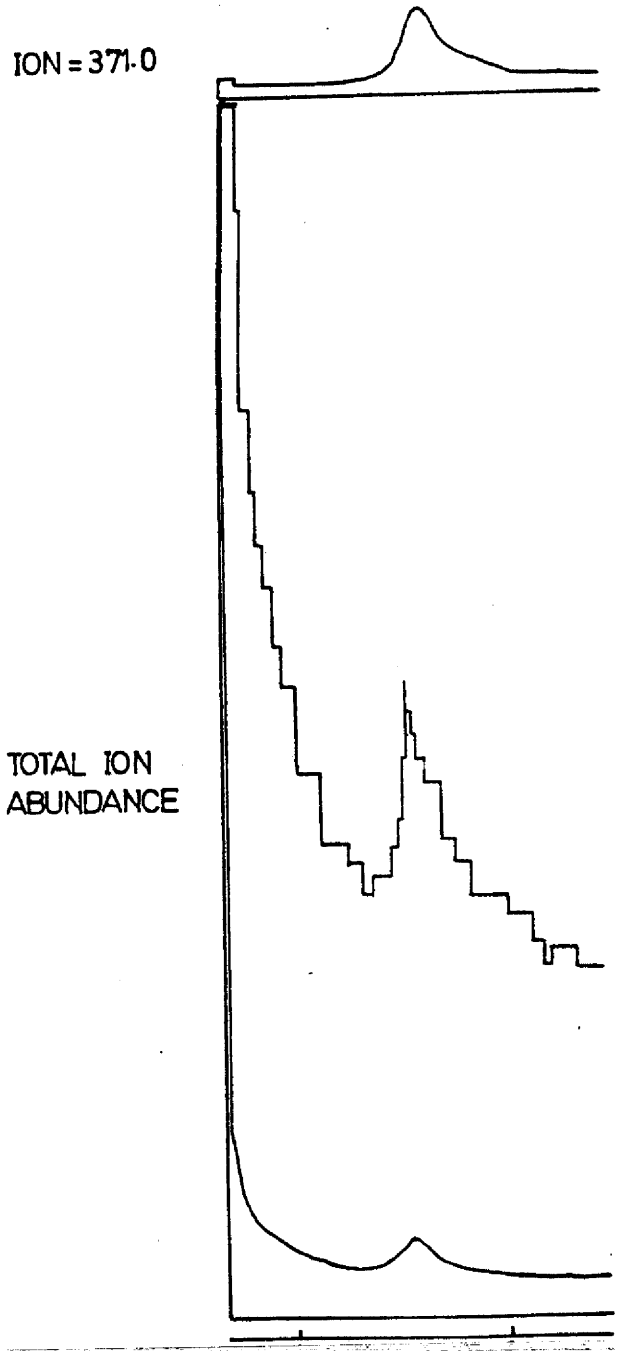
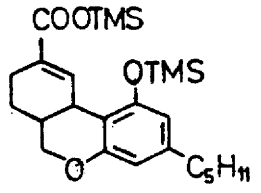
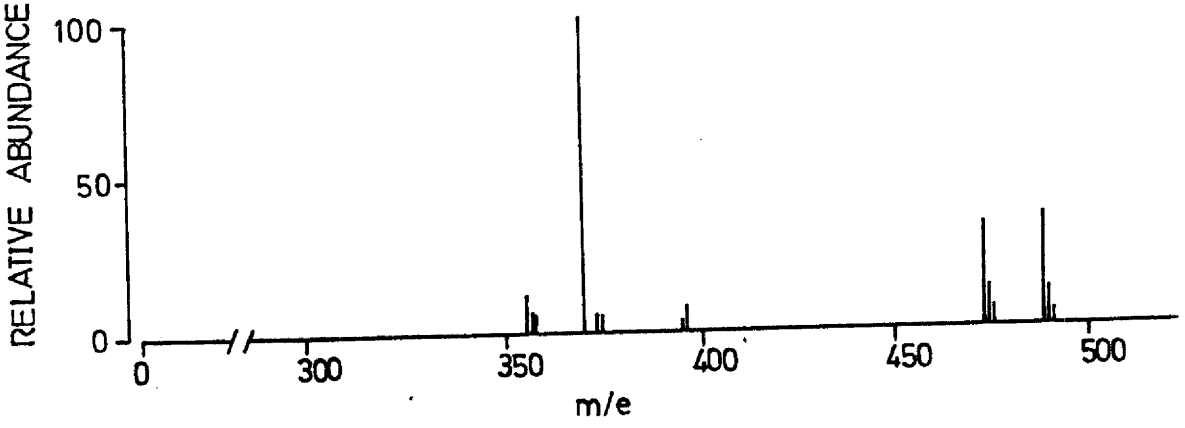


FIGURE 14 GCMS of THC-COOTMS

MASS	ABUNDANCE
370.6	100.0
473.3	31.6
488.3	34.9





tisation step rather than two. The silylation reagent chosen was BSTFA as it was readily available. Furthermore unlike BSA, the fluorinated by-products of the BSTFA reactions are volatile and thus do not interfere in the GC analysis.

The reaction conditions chosen (90°C for 90 minutes) ensure that silylation of the carboxyl function occurs. The success of the derivatisation procedure was demonstrated by GC and GCMS, using the bulk extract of THC-COOH which was subsequently cleaned-up by preparative TLC. Figure 13 shows the GC chromatogram of silylated THC-COOH. Figure 14 is a GCMS (PEAKFINDER mode) chromatogram and accompanying mass spectrum of the same sample. A further discussion on the GC and the GCMS work will be presented in the following two chapters, respectively.

CHAPTER 7

GAS CHROMATOGRAPHY

Gas chromatography is the main method used for the detection of drugs and their metabolites, for reasons of sensitivity and specificity. In cannabinoid analysis from biological fluids, GC together with GCMS make up the main confirmatory methods currently in use. The two main types of columns used are the methyl silicones and the phenyl methyl silicones. The common detectors are either an FID or the more sensitive ECD.

In this laboratory a GC procedure was developed using a methyl silicone column (OV-101) and FID detector. Both these items (OV-101 and FID) are the most commonly used combination in general laboratory drug analysis.

7.1 Instrumentation and Method

Gas chromatographic analysis was performed on a Model 5880 Hewlett-Packard gas chromatograph equipped with a flame ionisation detector. The column was a 6ft x 2mm i.d. silanised glass column packed with 3% OV-101 on 100/120 mesh Gas-Chrom Q, Applied Science Laboratories. The injector and detector were maintained at a temperature of 300°C. The column oven-temperature was programmed with nitrogen as the carrier gas at a flow rate of 20 ml min^{-1}. The initial oven temperature was set at 250°C and held for 10 minutes before being increased at a rate of 5°C min^{-1} until 280°C, and held at this temperature for 5 minutes. A sample volume of 1ul was manually

injected into the GC. A peak at an RT of 5.75 min to 5.80 min represented the silylated THC-COOH.

7.2 Discussion

A GC chromatogram of the TMS derivative of TLC-purified THC-COOH is illustrated in Figure 13, while Figure 15 shows that of a cannabis-containing urine extract (after silylation). The peak corresponding to the derivatised THC-COOH is shown in the figures.

In the GC of the extracts (Figure 15), the cannabinoid metabolite peak shows up clearly against a background of the other impurities.

The GC illustrated in Figure 15 represented the derivatised extract of a urine sample which gave an EMIT response just above that of the Medium Calibrator (75ng ml^{-1}). A semi-quantitative assessment of the cannabinoid concentration of the urine was 78ng ml^{-1}, based on the EMIT calibration curve.

The GC of the extract of a urine sample which gave an EMIT response slightly above that of the Low Calibrator (20 ng ml^{-1}) is shown Figure 16. As expected, the peak is smaller compared to the extract of the more concentrated urine sample of Figure 15. Nevertheless the peak is still distinctive as representing the TMS derivative of THC-COOH.

Another urine sample, with an EMIT response slightly lower than that of the Low Calibrator response, also gave a positive GC chromatogram for THC-COOTMS.

FIGURE 15

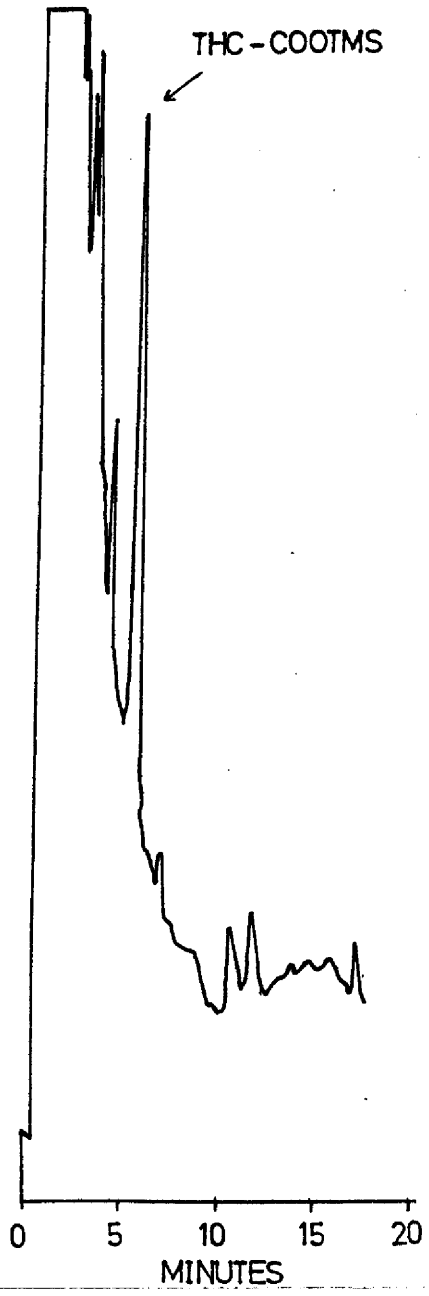


FIGURE 16

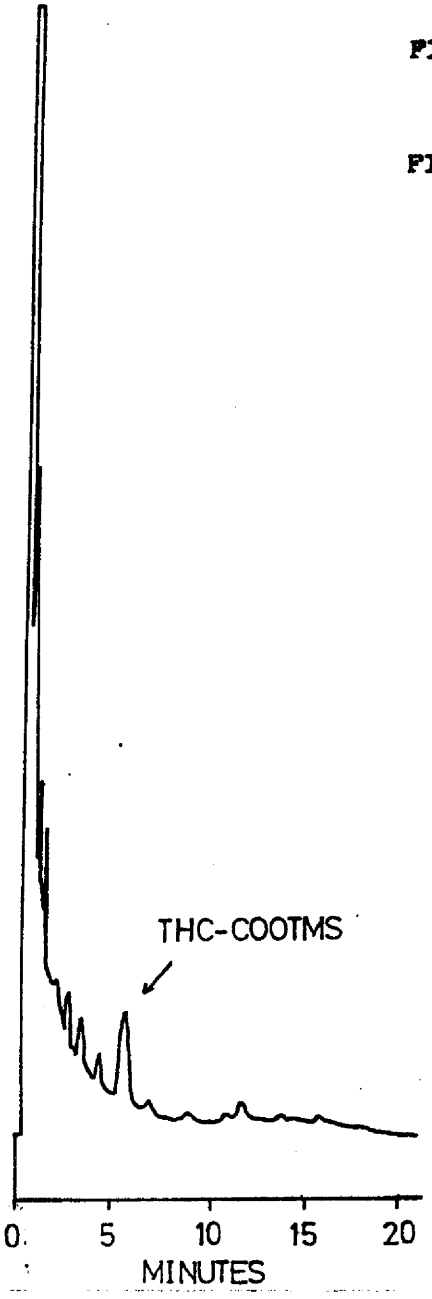


FIGURE 15 GC of URINE Extract (> 75 ng ml^{-1})

FIGURE 16 GC of URINE Extract (20 ng ml^{-1})

The other urine samples, which had EMIT responses beyond that of the Low Calibrator, showed the presence of THC-COOTMS by GC.

7.3 Conclusion

The results discussed above suggest that the GC procedure introduced here can be used as a confirmatory technique for urine samples giving an EMIT response equal to or greater than that of the Low Calibrator (20 ng ml^{-1}). This suggestion is made on the assumption that the EMIT set-up is available for screening purposes.

CHAPTER 8

GAS CHROMATOGRAPHY-MASS SPECTROMETRY

GCMS, by virtue of its selectivity and sensitivity, is the definitive analytical tool for the detection of trace quantities of drugs and metabolites. Whilst the GC component is capable of a high degree of chromatographic resolution of a mixture of components, the MS is capable of identifying each of the resolved components quite unequivocally. This identification is based on the mass spectrum of the compound in question. The mass spectrum yields information about the molecular weight (from the molecular ion peak) and about the chemical structure (from the fragmentation pattern). Together, the information is usually sufficient for correct identification.

The two most common modes of ionisation employed in drug analysis are electron impact (EI) and chemical ionisation (CI). GCMS utilising the former mode (GCEIMS) is less sensitive than the latter (GCCIMS). EIMS produces more fragmentation than CIMS and is therefore less sensitive for monitoring the molecular ion or base peak ion (selected ion monitoring, SIM).

In this laboratory GCMS was employed in the EI mode using an OV-101 packed glass column. GCMS studies were carried out both in the mass scanning and selected ion monitoring modes.

8.1 Instrumentation

GC/MS analysis was carried out on a model 5992 Hewlett-Packard GC/MS. The column was a 2ft x 2mm i.d. silanised glass column packed with 3% OV-101 on 100/120 mesh Gas-Chrom Q. The injector was maintained at 280°C. Helium was the carrier gas at a flow rate of 20 ml min^{-1}.

The oven temperature was programmed to be maintained at an initial value of 250°C for 2 minutes before rising at 10°C min^{-1} to 280°C, and maintained at this temperature for a further 2 minutes.

The MS detector was used to monitor the GC eluate in either the PEAKFINDER mode or the SIM mode. In the Peakfinder mode, the MS settings were as follows:-

SOLVENT ELUTE TIME:	0.8 min
MS PEAK DETECT THRESHOLD:	500
SAMPLES PER 0.1amu:	2
ELECTRON MULTIPLIER VOLTAGE:	2,600
GC PEAK DETECT THRESHOLD:	100
MASS SCAN RANGE:	300-500 amu
SINGLE ION TRACE:	489 amu (M^+) or 371 amu (base peak)

In the SIM mode the MS settings were:

SOLVENT ELUTE TIME:	0.9 min
ELECTRON MULTIPLIER VOLTAGE:	2,600
ION MASS MONITORED:	371 amu (Base peak)
DWELL TIME:	1,000 ms
SIM WINDOW SIZE:	1 amu

The MS was self calibrated each day using the AUTOTUNE facility provided. A sample volume of ul was used. The retention time for the TMS derivative of THC-COOH is 1.5 min.

8.2 Discussion

Figure 14 shows the GC chromatogram and the MS fragmentation pattern of TLC-purified THC-COOTMS recorded after GCMS analysis. The upper trace of the GC chromatogram monitors only the m/e 371 ion while the lower trace represents the total ion abundance of the eluted component. The m/e 371 ion represents the base peak of THC-COOTMS (M^+489). Monitoring the base peak is a sensitive method of following the elution of a component in GCMS.

The base peak (m/e 371, see fragmentation pattern in Figure 14) represents a loss of 117 amu from the moleular ion (M^+489). This loss is accounted for by the facile loss of the carboxy-TMS ester function (-COOTMS). A less pronounced fragmentation at 15 amu below the M^+ is due to loss of a methyl function, most likely from the cannabinoid ring nucleus.

In Figure 14 the component eluting at RT=1.5 minutes thus represents the TMS derivative of THC-COOH, by virtue of its mass spectrum.

Having now established the RT and MS of THC-COOTMS, the sensivity of the system may be increased by performing GCMS analyses in the SIM mode. In this mode, one ion (or up to six ions for specificity) is monitored continuously during the GCMS run. The ion of choice would be the base peak ion (371 amu) for enhanced sensitivity. In the SIM mode no mass spectrum is available since no scanning is undertaken.

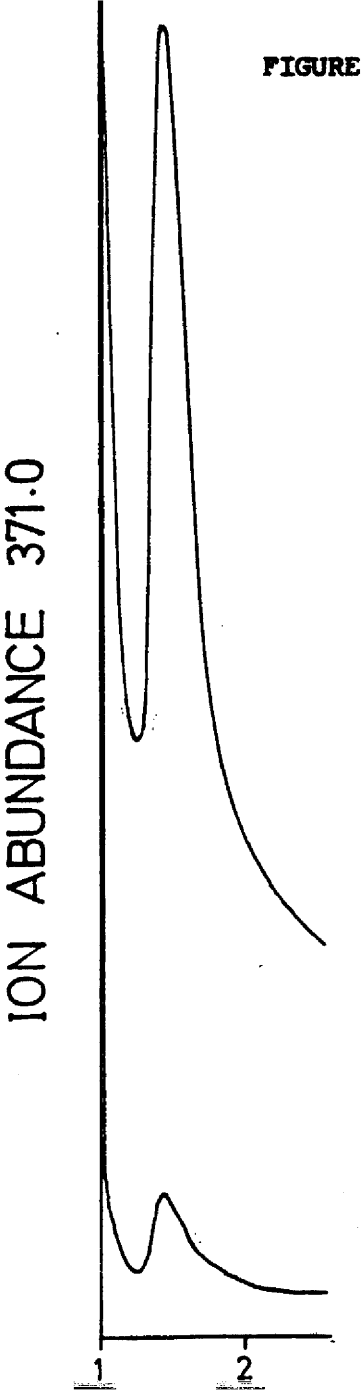
A sampling of urine extracts that were used in the GC study was run through the GCMS in the SIM mode (single ion at m/e 371). Figure

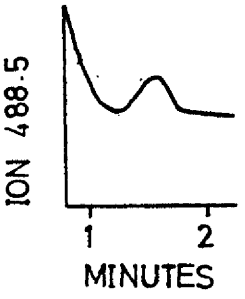
FIGURE 17 **GCMS of URINE Extract**

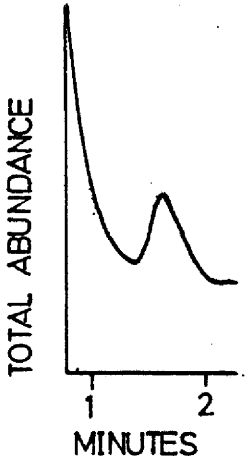
a. > 75ng ml^{-1} THC-COOH

b. 20ng ml^{-1} THC-COOH

MASS	ABUNDANCE	DWELL TIME
488·5	8·43	500 ms
371·0	24·12	500ms







17a represents the GC chromatogram of the derivatised extract of a urine sample which gave an EMIT response slightly greater than that of the Medium Calibrator (75 ng ml^{-1}). Figure 17b shows a similar chromatogram of a urine sample having an EMIT response equal to that of the Low Calibrator (20 ng ml^{-1}).

8.3 Conclusion

The preceding section has demonstrated the ability of the GCMS procedure developed here to detect THC-COOH in urine samples giving an EMIT response equivalent to 20 ng ml^{-1}, which is equivalent to the EMIT Low Calibrator response (cut-off level for EMIT).

CHAPTER 9

ENZYME-MULTIPLIED IMMUNOASSAY TECHNIQUE

The enzyme multiplied immunoassay technique, EMIT is a relatively recent immunological method for the detection of drugs of abuse in urine. It is based on the principle of competitive enzyme inhibition by an antibody for the hapten-labelled enzyme and the free drug in the urine.

The EMIT kit is provided with three Calibrators - Negative (0 ng ml^{-1} of the THC-COOH), Low (20 ng ml^{-1}) and Medium (75 ng ml^{-1}). Only urine sample with an EMIT response equal to or above the Low Calibrator response are considered positive for cannabis. The Low Calibrator response is appropriately termed the "cut-off point".

Using the 3 Calibrators provided, a calibaration curve of EMIT response vs calibrator concentration can be plotted (Figure 11). With this curve, the cannabinoid concentration of unknown urine samples can be estimated.

Apart from the calibrators, a Cannabinoid Assay Kit is also provided. This kid comprises (after reconstitution with water) a buffer and two immunochemical reagents (A and B).

9.1 Materials

The EMIT Cannabinoid Calibrator Kit and EMIT Cannabinoid Assay Kit were obtained for Syva Co. (USA). The EMIT Cannabinoid Kit consists of lyophilised urine:-

i. Negative Calibrator 0 ng ml^{-1} (3 ml when reconstituted)
ii. Low Calibrator 20 ng ml^{-1}
iii. Medium Calibrator 75 ng ml^{-1}

The EMIT Cannabinoid Assay Kit, when reconstituted, consists of:-

i. Reagent A: Antibody to the drug and enzyme (NADH) 0.15 M glycine buffer at pH5 (8ml)

ii. Reagent B: Enzyme labelled drug (MDH-THC), stabilisers and preservatives in 0.1 M Tris-HCl buffer at pH 7.4 (6 ml)

iii. Buffer: 0.15 M Tris pH 8.9 buffer containing the enzyme substrate (maleate) and preservatives

After reconstitution, the reagents and calibrators should be allowed to equilibrate for at least 4 hours at room temperature or overnight at 4°C before use. They should not be frozen or exposed to temperatures above 32°C.

If stored in the fridge, the reagents and calibrators should be allowed to reach room temperature before use. The reagents are stable for up to 12 weeks after reconstitution, after which they have to be discarded. The calibrators are stable for only 2 weeks if stored at 4°C. Urine samples used for the experiment were obtained from users. Methodone, morphine, amphetamine, phenobarbital and THC were obtained as pure standards from the UNDND, Vienna.

9.2 Instrumentation

The necessary instrumentation for the EMIT Cannabinoid procedure which were supplied by SYVA Co, together with their respective settings are listed below. It is called the Syva Lab 5000 Plus Instrument System.

	INSTRUMENT	FUNCTION	SETTINGS
1.	SYVA S-III	Spectrophotometer	Wavelength: 340nm Mode: Concentration Con.Cal.: Amplification 2.667 Temperature: 30°C Sample Vol.: 0.5 ml. Sample time: 0.3 sec.
2.	SYVA CP-500 EMIT CLINICAL PROCESSOR	Data Handling	Program: SET TIMES Delay: 15 sec. Measure T: 30 sec.
3.	SYVA PIPETTER DILUTER	Sample Handling	Sample Vol.: 50 ul Delivery Vol.: 50 ul sample + 250 ul buffer

9.3 Assay Procedure

50 ul of urine sample together with 250 ul of EMIT buffer are mixed with 50 ul of Reagent A and 250 ul of EMIT buffer in a beaker. The mixture is allowed to equilibrate for at least 30 seconds, whereupon 50 ul of Reagent B and 250 ul of buffer are added. The contents of the beaker is immediately aspirated into the spectrophotometer flow-cell. The printer to time and record the measurement is automatically activated. On completion of the measurement, the mixture is aspirated to waste.

While the first sample is being measured, another sample may be prepared. A suggested flow diagram of the EMIT procedure is shown in Figure 18.

To meet the performance standards and eliminate the possibility of false positive results, a BLANK RUN has to be carried out for any sample that yields a positive response. Before running a blank, the flow cell of the spectrophotometer has to be flushed with distilled water and the pipetter-diluter flushed with cannabinoid buffer solution. A blank run is performed by mixing 50 ul of sample and 250 ul of buffer with 50 ul of Reagent A and 250 ul of buffer. This is followed by adding 300 ul of buffer and aspirating the total into the spectrophotometer.

A blank reading of less than 10 units can be safely ignored. For values above 10, the reading is to be subtracted from the sample reading and the new value considered.

After use the flow cell and pippeter-diluter should be cleaned as recommended by the manufacturer.

9.4 Sample Preparation

If the urine samples are not to be analysed the same day they should be stored frozen until required. On thawing, any precipitate formed should be removed by centrifugation. The same applies to all urine samples that appear turbid.

The pH of the urine samples for EMIT analysis have to be in the range 5.5 to 8.0 for proper interpretation of results. The pH may be brought to within this range by the addition of 1 M HCl or 1 M NaOH.

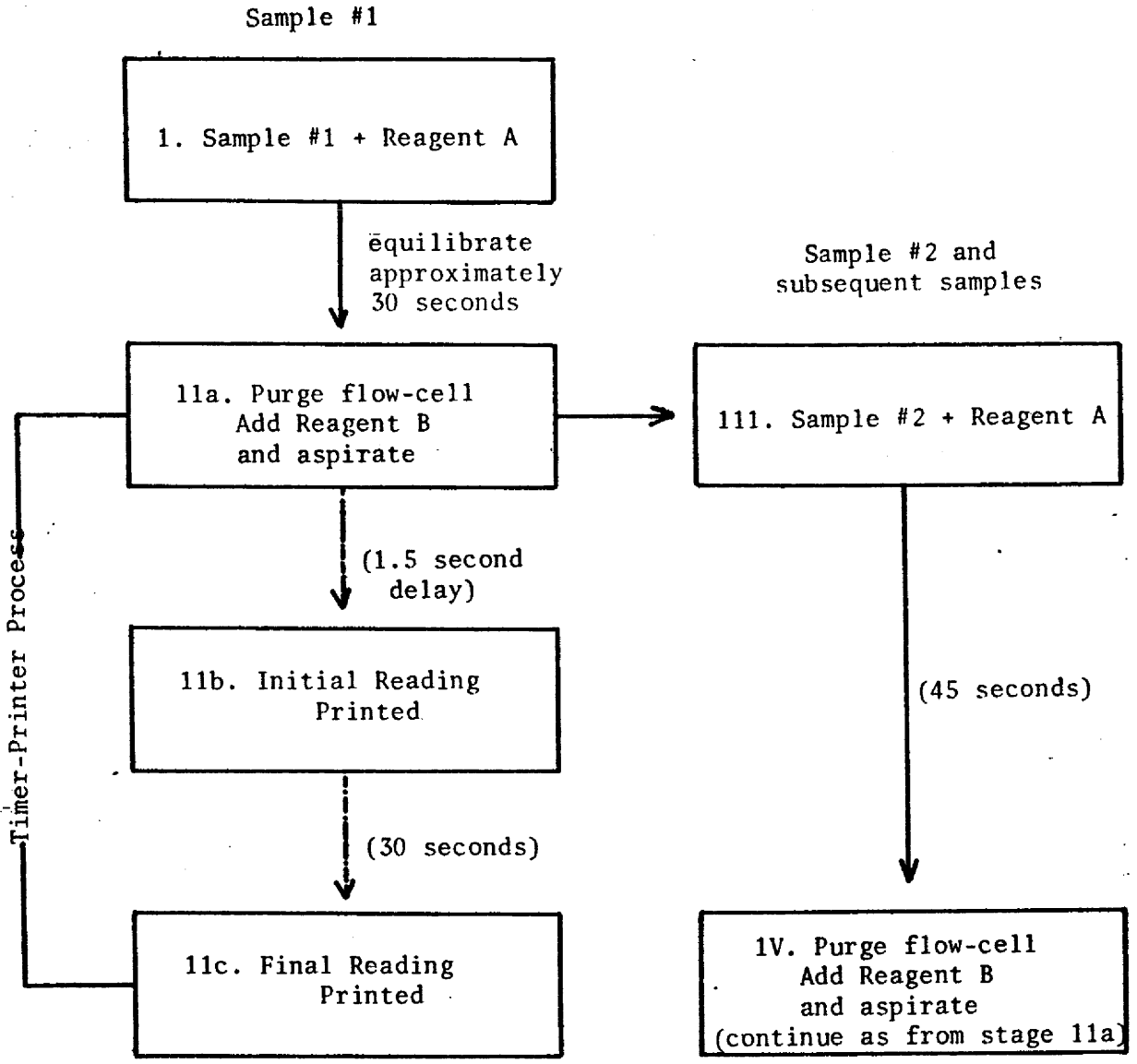


Figure 18

A Suggested Working Procedure for the EMIT Cannabinoid Assay

The samples should also not be unnecessarily exposed to direct sunlight or high temperature.

9.5 Methods

A. Interpretation of Results

After aspirating the sample mixture into the spectrophotometer flow cell, two absorbance readings are automatically made on each sample. The first is at 15 seconds and the second at 45 seconds. The difference between these two readings is printed out (ΔA or EMIT response value). If the ΔA value of a particular sample is equal to or above that of the standard Low Calibrator, then the sample can be considered positive for cannabinoids, provided a blank has also been run.

Conversely, an EMIT response less than the Low Calibrator can be considered negative. No blank run needs to be carried out.

B. Cross-Reactivity Studies

Blank urine samples were spiked with varying amounts of the following drugs, up to a concentration of 1 mg ml^{-1}:-

Morphine

Phenobarbitone

Methadone

Amphetamine

The samples were then tested by EMIT using the Cannabinoid Assay protocol.

C. Sensitivity of EMIT to THC

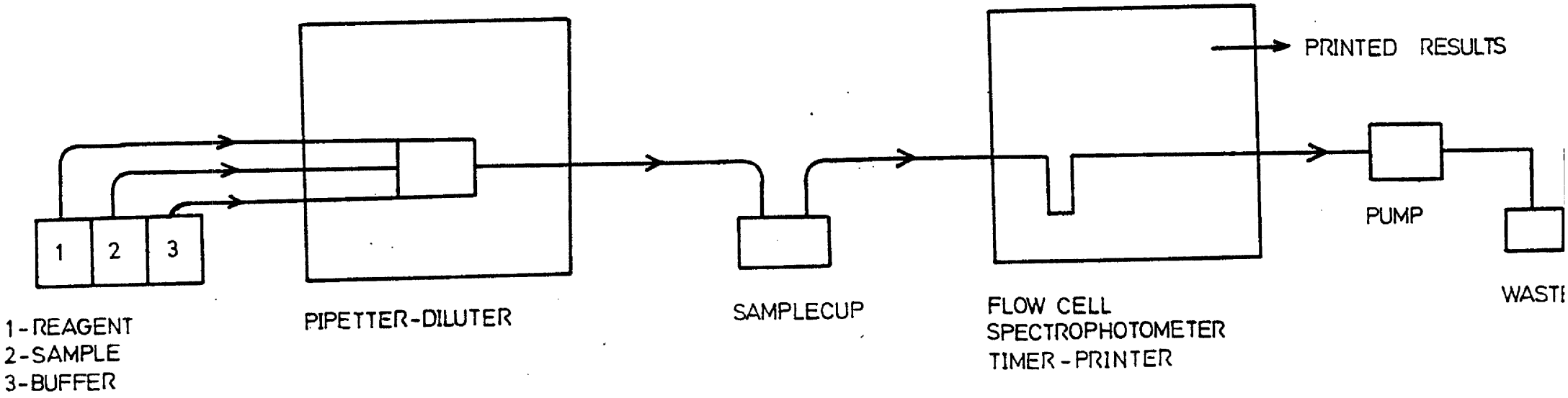
Spiked urine samples containing 0-400 ng ml^{-1} of THC were subjected to the EMIT Cannabinoid Assay procedure. A calibration curve of EMIT response vs Log.(THC) was plotted.

9.6 Discussion

The operation of the assay procedure for analysing samples is relatively straight forward and can be learnt in half an hour. With the procedure it is possible to prepare the following sample while the first sample is being measured. A procedure suggested by the manufacturers employing this technique is presented in Figure 18. Figure 19 illustrates the sequence of events involved at the various points of the EMIT instrument during the measurement.

A batch of cannabinoid urine samples were subjected to the EMIT procedure. One sample gave an EMIT response slightly lower than the Low Calibrator (20 ng ml^{-1}) reading. Six samples gave EMIT values between the Low and Medium Calibrator (75 ng ml-1) readings. Five samples gave EMIT responses above the Medium Calibrator Value (Table 3).

When these urine samples were extracted, derivatised and chromatographed following the procedure developed in this laboratory, all the samples showed positive for THC-COOH by GC and GC-MS. Only the samples which gave a response greater than the Medium Calibrator were positive by TLC. Of these six samples, three gave faint spots on TLC. These samples had EMIT readings close to the Medium Calibrator response. The results are summarised in Table 3.



EMIT CANNABINOID ASSAY INSTRUMENTS

FIGURE 19

TABLE 3

Comparison of the EMIT Response of Urine Samples with TLC, GC and GCMS

METHOD	EMIT CALIBRATOR RESPONSE NUMBER OF URINE SAMPLES					
	< LOW (<20 ng ml^{-1})		LOW - MEDIUM (20-75 ng ml^{-1})		>MEDIUM (>75 ng ml^{-1})	
	+ve	-ve	+ve	-ve	+ve	-ve
EMIT	0	1	5	0	6	0
TLC	0	1	0	5	6	0
GC	1	0	5	0	6	0
GCMS	1	0	5	0	6	0

The single EMIT-negative sample that showed positive by GC and GCMS had an EMIT response very close to the cut-off Low Calibrator reading.

The samples below the Medium Calibrator readings were found negative by TLC because of the inherent lack of sensitivity of this technique.

The preliminary cross-reactivity studies with morphine, methadone, amphetamine and phenobarbitone showed the absence of any cross-reactivity even at the high concentration of 1 mg ml^{-1} used.

The sensitivity of the EMIT cannabinoid assay to THC was tested by running THC-spiked urine samples. A plot of EMIT response vs log (THC) is shown in Figure 20, together with an EMIT Calibration Curve.

From Figure 20, the THC concentration which elicits an EMIT response corresponding to the cut-off Low Calibrator is 67 ng ml^{-1}. This value is close to the manufacturer's quoted figure of 70 ng ml^{-1}.

The EMIT instruments were functioning properly throughout the study. However the spectrophotometer takes about 45 minutes to stabilise when switched on (the manufacturers recommend a warm-up time of 30 minutes). After this initial period, no significant drift in the absorbance display was observed during the course of each experiment. The manufacturers state that any persistent drift after the initial warm-up period may be attributed to a dirty flow cell. A cleaning procedure is available from the manufacturer.

The EMIT technique and the observed readings are operator dependent to a certain extent because of the time taken between mixing the

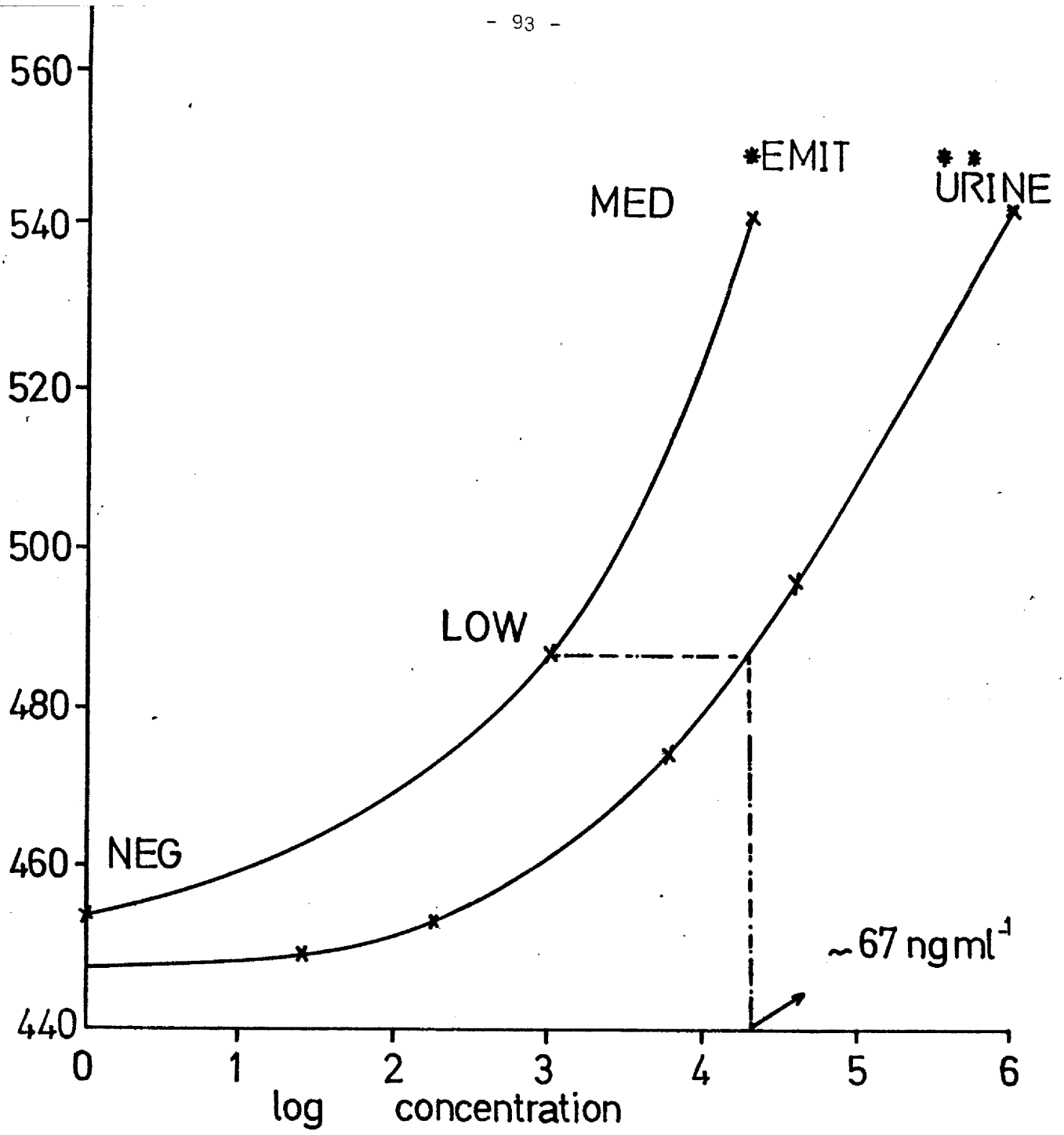


FIGURE 20 **EMIT Response Curve For THC**

final reagent (Reagent B) and transferring the mixture to the spectrophotometer unit for aspiration. As soon as Reagent B is added, the enzymic reaction commences. Since the reaction rate is only measured spectrophotometrically from the time the mixture is aspirated into the flow-cell, it is essential that the transferrence time be kept constant. This is easily achieved if the same operator performs the assay.

9.7 Conclusion

The EMIT cannabinoid assay has been demonstrated not to cross-react with morphine, methadone, amphetamine and phenobarbitone even at a high concentration of 1 mg ml^{-1}.

The sensitivity of the EMIT assay to THC was found to be lower than THC-COOH (67 ng ml^{-1}) as claimed by the manufacturers.

With the limited number of urine samples that were tested, no false-positives were recorded. The only false-negative sample had an EMIT response close to the cut-off point.

The EMIT system appears to be a suitable method for the screening of urine samples for cannabinoids. The analysed samples could be divided into 3 groups:-

Group A: Samples with an EMIT response less than the Low Calibrator;

Group B: Samples with an EMIT response between the Low and Medium Calibrators;

Group C: Samples with an EMIT response above the Medium Calibrator.

If 20 ng ml^{-1} is taken to be the cut-off point for samples that are to be considered either positive or negative then the above groups of samples should be treated as follows:-

Group A: Samples are to be considered negative and not tested further;

Group B: The samples should be confirmed by either GC or GCMS. Alternatively they may be double-checked using both techniques. TLC is not sufficiently sensitive.

Group C: These samples need to be confirmed by TLC above.

It is most likely that samples giving a response greater than the Medium Calibrator are definitely cannabinoid-containing and so a confirmatory technique might not be necessary.

It should be noted that the EMIT procedure measures the presence of the cannabinoid metabolites collectively. The chromatographic-techniques, however, separate and identify only the major cannabinoid metabolite, THC-COOH. So it is conceivable that a urine sample may give a positive EMIT response but be found to be negative by GC or GCMS. However such a situation seems unlikely because even at 20 ng ml^{-1} (the cut-off point) the amount of THC-COOH present would be sufficient for detection by GC or GCMS.

Another factor that has to be considered is that the sensitivity in terms of ng ml^{-1} of original urine of the chromatographic methods

increases with increasing volume of urine used, as the amount of cannabinoids extracted would be greater and therefore more detectable. However, increasing the volume by too large an amount might pose problems of handling during the extraction procedure. Furthermore, on some occasions, large sample volumes might not be available for analysis.

Recently the manufacturers, Syva, have introduced a new Low Calibrator set at 100 ng ml^{-1} and a Medium Calibrator at 400 ng ml^{-1}. With such a calibrator, the effective detectability of the EMIT would be reduced from the current 20 ng ml^{-1} to 100 ng ml^{-1}. With the adoption of the 100 ng ml^{-1} cut-off a confirmatory technique (if at all necessary) such as TLC would be sufficient.

This new Cannabinoid assay is based on the enzyme Glucose-6-Phosphate Dehydrogenase (G6PDH) and is called the "DAU Cannabinoid-100 Assay" (DAU = drugs of abuse in urine; 100 = 100 ng ml^{-1} cut-off). The manufacturers intend to retain the present cannabinoid assay which uses the enzyme Malate Dehydrogenaze (MDH), now known as the "DAU Cannabinoid - 20 Assay" (20 = 20 ng ml^{-1} cut-off).

The manufacturers claim the new cannabinoid assay to have the following advantages:-

i) A simple assay procedure

ii) Shorter equilibration time - the reagents can be used an hour after reconstitution

iii) No blank runs after each positive sample

iv) Reagent stability - the Calibrators are stable up to 12 weeks

v) Uniform assay protocol with other DAU procedures.

The main advantages of the EMIT system for screening is its speed and relative sensitivity. Forty samples can be analysed per hour by a single operator. However, the Cannabinoid Assay Kit costs approximately M$600 and the Cannabinoid Calibrators M$100. The Assay Kit can be used to test 100 samples. Thus the reagent cost per sample is about M$7.

CHAPTER 10

EXTRACTION AND DERIVATISATION EFFICIENCIES

The two main steps in the analytical procedure for the detection of THC-COOH in urine are the extraction and derivatisation steps. To ensure maximum sensitivity of the procedure it is necessary that the above steps be optimised to ensure that the recovery at each stage is as high as possible.

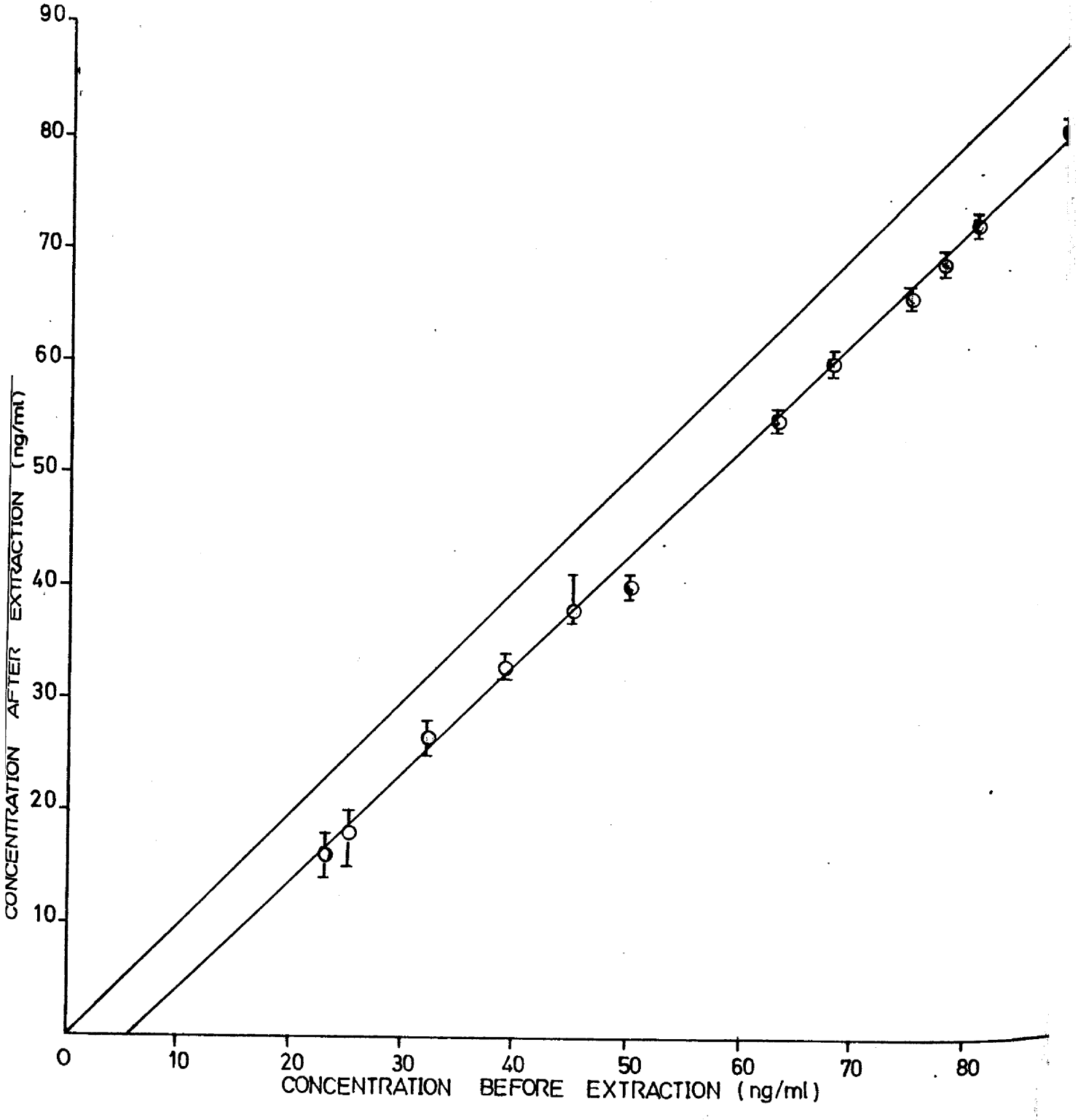
An estimate of the losses incurred during the extraction and derivatisation stages is presented here.

10.1 Extraction Efficiency

10.1.i Method

The cannabinoid concentration of urine samples (in the range 0 - 100 ng ml^{-1}) was estimated by the EMIT semi-quantitative procedure discussed previously. Each sample (in triplicate) was subjected to the extraction procedure developed here (see Chapter 4). The dried extracted residue from each sample was reconstituted in 2 ml (the original volume of urine used in this experiment) distilled water (adjusted to pH 7.5). The EMIT response of the reconstituted samples were measured and compared with the original readings. A graph of cannabinoid concentration before extraction vs after extraction is plotted (Figure 21).

FIGURE 21 Graph of THC-COOH Concentration After vs Before Extraction



10.1.ii Discussion

There are two plots in Figure 21. The line passing through the origin with a slope of 1 (angle of 45° to either axis) represents the ideal situation where there is no loss during the extraction procedure.

The second plot derived from the experimental data is linear and intersects the x-axis at 5.5 ng ml^{-1} and is at a slight angle to the theoretical line.

The plot shows the existence of a minimum loss corresponding to 5.5 ng ml^{-1}, and that this loss increases slightly with increasing concentration. Inquantitative terms, the loss of a 20 ng ml^{-1} sample is estimated (from Figure 21) at 6 ng ml^{-1} whilst the loss of a 100 ng ml^{-1} sample is estimated at only 9 ng ml^{-1}. The difference in the two is relatively small over such a concentration range.

The losses incurred could be attributed to the following factors:-

i. adsorptive losses onto glass surface;
ii. hydrolytic losses;
iii. losses during handling of the organic extract;
iv. apparent loss due to the removal of non-acidic cannabinoids during the extraction (eg. THC-OH) which give a response by EMIT.

The first factor (adsorptive losses) is independent of cannabinoid concentration, but dependent on the glass surface

area of the vessels used. However, since all glassware was silanised prior to use, such losses would be minimal.

The second factor (hydrolytic losses) would also be fairly insubstantial as the conditions used (50°C, 30 minutes) were relatively mild. Conversely it may be that the reaction conditions were too mild, resulting in incomplete de-conjugation of THC-COOH from its glucuronide moiety. The bound THC-COOH would be lost in the extraction process. Increasing the temperature of the hydrolytic process did not improve the yield. This suggests that incomplete deconjugation of THC-COOH is not the likely reason for the observed loss. In either case, the loss would be dependent on concentration.

The third factor (handling losses) could contribute to the observed losses. During the transference of the organic phase (organic extract) from the urine-containing vessel for subsequent evaporation, a small volume of the extract is left behind (interphase region). This layer is left to prevent the transfer of any of the aqueous phase (urine) into the organic extract. The loss incurred here would therefore be dependent on the concentration of the sample. This loss can be reduced by diluting the interphase with organic solvent and repeating the transference (c.f. double extraction).

The fourth factor (non-acidic cannabinoid loss) is also quite likely as the extraction process here only retains the acidic cannabinoids (principally THC-COOH). The other neutral

cannabinoids (mainly THC-OH) are lost. Thus, whereas the original EMIT reading will register the presence of these latter cannabinoids, the EMIT response of the reconstituted urine extract will note their absence. This apparent loss, which is a function of the initial cannabinoid concentration, is an inherent aspect of the extraction process.

Conclusion

The extraction procedure employed here is subject to losses which are considered small. The minimum loss is 5.5 ng ml^{-1}. In actual terms this loss is translated to a 6 ng ml^{-1} loss for a urine sample giving a EMIT response equivalent to 20 ng ml^{-1} and an 8 ng ml^{-1} loss for a sample corresponding to 75 ng ml^{-1} by EMIT.

The loss is dependent on concentration and the two most likely contributory factors are:

i. loss of THC-COOH during handling of the organic extract, and

ii. loss of THC-OH and other neutral cannabinoids during the extraction steps.

II. Derivatisation Efficiency

Method

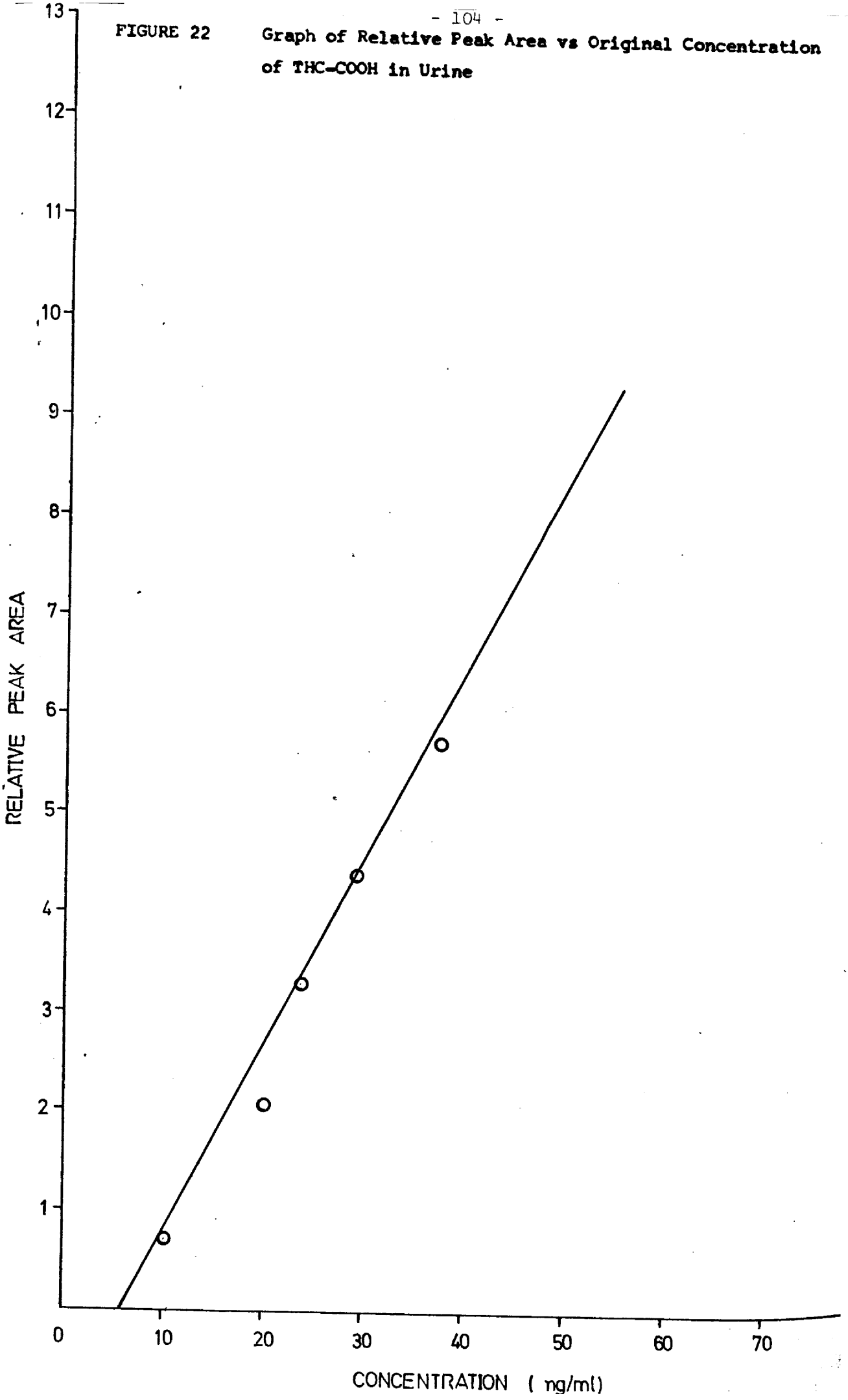
The cannabinoid concentration of urine samples containing varying amounts (0-100 ng ml^{-1}) of cannabis was estimated by EMIT. The samples were then subjected to the extraction and derivatisation procedure developed in this laboratory (see earlier chapters). The samples were analysed by GC and a graph of relative peak height vs sample concentration (before derivatisation) was plotted (Fig. 22). The plot was obtained by substracting the extracting losses from the experimental data.

Discussion

In the graph of relative peak height vs sample concentration shown in Figure 22, the intercept of the plot at the x-axis is at 5.0 ng ml^{-1}. This suggests a minimum loss of 5 ng ml for the post-extraction prosess. The post-extraction steps include the transfer of the redissolved (small volume) extract to a microvial, evaporation of the solvent and derivatisation.

Some losses may be expected during the transfer of the concentrated extract solution to the microvial. A small amount of the extract may be left behind in the original vessel (test-tube) or in the pipette used in the transfer. These losses are kept to a minimum by washing out the vessel and repeating the transfer using a small volume of solvent. The losses mentioned here would be concentration dependent.

FIGURE 22 Graph of Relative Peak Area vs Original Concentration of THC-COOH in Urine



Again, concentration-independent loss due to adsorption on glass is possible, although this would be insignificant because of the silanisation process already discussed.

Incomplete derivatisation due to insufficient BSTFA cannot be accepted because of the large excess of the silylating agent used. Furthermore, the reaction conditions are sufficiently strong to allow complete derivatisation.

One likely possibility is the steric factor. Because of the spatial proximity of the two functional groups to be derivatised, coupled with the size of the TMS group and BSTFA it is conceivable that silylation at one position sterically hinders derivatisation at the second position. In the case of THC-COOH, the phenolic OH gets derivatised first which then retards silylation of the carboxyl group. This should not affect the percentage yield.

Conclusion

The total minimum loss of the extraction and derivatisation procedure is 10.5 ng ml^{-1}. This loss can be divided into losses during extraction (5.5 ng ml^{-1}) and the post-extractive and derivatisation steps (5.0 ng ml^{-1}). Furthermore the loss during the extraction procedure increases marginally with increasing concentrations (6 ng ml^{-1} at 20 ng ml^{-1} and 8 ng ml^{-1} at 75 ng ml^{-1}). Various possibilities have been cited for the observed losses during both stages the procedure.

CHAPTER II

CONCLUSION

In addition to the presentation of suitable analytical procedures (TLC, GC and GCMS) for the detection of THC-COOH in urine, this report also includes a review of the existing methods being used in other laboratories.

The chromatographic procedures developed here require a relatively small volume (3ml) of urine for analysis. The sensitivities of these procedures are sufficient for the detection of THC-COOH in urine of a recent cannabis consumer. The sensitivity of the TLC procedure is about 75 ng ml^{-1} and those of GC and GCMS are about 20 ng ml^{-1}.

The minimum losses incurred during the extraction and post-extraction (derivatisation) stages are 5.5 ng ml^{-1} and 5 ng ml^{-1} respectively. These losses can be attributed to handling (mainly during solvent transfer) procedure and stereochemical (steric hidrance to derivatisation) factors.

The EMIT procedure for the detection of cannabinoids in urine was tested against the chromatographic procedures mentioned above. With the limited number of test urine samples available, a good correlation existed between these methods. Samples which gave an EMIT response between 20 ng ml^{-1} and 75 ng ml^{-1} were confirmed by GC and GCMS. Samples with an EMIT response greater than 75 ng ml^{-1} were confirmed by TLC, GC and GCMS.

Due to the absence of any sample preparation for EMIT analysis, together with its rapid analysis time, it is suggested that EMIT be used as a screening procedure. Any samples with a response in the 20-75 ng ml^{-1} range can be confirmed by either GC or GCMS. Samples giving an EMIT response above 75 ng ml^{-1} can be confirmed by TLC.

Table 4 lists the estimated start-up cost as well as running cost per sample for each of the above analytical procedures. It is evident that the start-up cost of the EMIT and the GC are comparable ($30,000) with the TLC being considerably lower ($2,000) and the GCMS very much higher. The personnel cost for all the methods are comparable, the differences being seniority (JLA vs LA vs SLA vs CT). The reagent cost for the EMIT is substantially higher ($2,100) compared to other methods. This is because the Cannabinoid Kits are relatively expensive. The consummables cost for TLC refers to the use of commercial precoated plates as opposed to the less costly lab-coated variety. For GC and GCMS, the gases consumed are minimal.

The sample turnover of EMIT and TLC is greater than that of GC or GCMS (30 per day). These figures are based on running 40 samples per hour on EMIT, 10 samples per TLC plate (4 plates per hour, run simultaneously), and 4 samples per hour on either GC or GCMS.

A high sample turnover is useful for screening purposes where a large number of samples have to be analysed. Both the EMIT and TLC are therefore candidates for screening purposes. The EMIT however is the more suitable screening method for the following reasons:-

TABLE 4

Cost Comparison of the Different Cannabinoid Analytical Procedures

	EMIT ($)	TLC ($)	GC ($)	GCMS ($)
Start-up cost	30,000	2,000	30,000	300,000
Personnel cost per day	10	10	15	20
Reagent cost per day	2,100	150	5	5
Sample turnover per day	300	100	20	20
Running cost per sample	7	0.50	0.75	0.75

NB: The service and maintainance costs of the GC and GCMS are not included in the figures above.

i. no sample preparation is necessary (no extraction or derivatisation);

ii. a very small sample volume is required (100 ul);

iii. a short analysis time (1½ minutes);

iv. reasonable sensitivity (20 ng ml^{-1}) compared to TLC (75 ng ml^{-1}).

v. capable of being upgraded (extra $20,000) to a fully automated system capable of analysing up to 60 samples per hour

The factors in favour of TLC are mainly monetary:-

i. lower start-up cost ($2,000 vs $30,000 for EMIT);

ii. lower running cost per sample ($0.50 vs $7.00 for EMIT).

A choice between the two would therefore eventually rest on the constraints imposed on the laboratory which are mainly:-

i. financial

Further work is currently being carried out in this laboratory with the aim of reducing the losses incurred during the extraction and derivatisation procedure with a view to improve the present detection limits. Proposed work to study the pharmokinetic aspects of cannabis consumption will be undertaken.

CHAPTER 12

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